THE EFFICACY OF DIAVITE™ (*PROSOPIS GLANDULOSA*) AS ANTI-DIABETIC TREATMENT IN RAT MODELS OF STREPTOZOTOCIN-INDUCED TYPE 1 DIABETES AND DIET-INDUCED-OBESE INSULIN RESISTANCE

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DECLARATION:

I, the undersigned, hereby declare that the work contained in this dissertation is my original work and that I have not previously, in its entirety or part, submitted it at any university for a degree.

Signature: .....

Date: .....

Abstract

Introduction: Obesity and its associated complications, such as the metabolic syndrome, hypertension and cardiovascular disease, are escalating worldwide. In recognition of this, untested remedies advertised as anti-diabetic agents are flooding the market. Many of these products have limited efficacy, limited tolerability and significant side-effects. One remedy, claiming to have anti-diabetic properties, is Diavite^{TM}. Diavite^{TM}, a herbal product, consisting solely of the dried and ground pods of the *Prosopis glandulosa* tree, which is currently marketed as a food supplement with blood glucose and blood pressure stabilizing properties, as well as having the ability to enhance glucose utilization. It is already freely available from agents as well as sold over the counter at pharmacies. The producers of Diavite^{TM} are now seeking registration for their product from the Medicines Control Council (MCC) and, therefore, require solid scientific evidence of its effects.

Aims: The aims of our study were, on request of the producing company, to determine the efficacy of Diavite^{TM} (*P. glandulosa*) as an anti-diabetic agent and possible mechanisms of action of this plant product.

Methology: We utilized rat models of streptozotocin (STZ)-induced type 1 diabetes and diet-induced obese (DIO) insulin resistance. Male Wistar rats were rendered (a) type 1 diabetic after a once-off intra-peritoneal injection of STZ at a dose of 40 mg/kg and (b) insulin resistant after being on a high caloric diet (DIO) for 16 weeks. Half the animals of the type 1 diabetes model as well as the insulin resistant model were placed on Diavite^{TM} treatment (25 mg/kg/day) for a period of 4 – 8 weeks, depending on the model. The STZ-induced type 1 diabetic rats were sacrificed and the pancreata harvested for histological analysis. Animals on the DIO diet were sacrificed and (i) intra-peritoneal fat weight determined (ii) isolated hearts subjected to ischaemia/reperfusion to determine infarct size and protein expression profiles and (iii) cardiomyocytes prepared to determine insulin sensitivity. At the time of sacrifice blood was collected for blood glucose and serum insulin level

determination, for both models. In addition, a standard toxicology study was performed in Vervet monkeys over a 3 month period.

Results: In our type 1 diabetic model (blood glucose > 10 mmol/L) with a β-cell reserve, Diavite™ treatment lead to increased serum insulin levels ($p < 0.001$) in both control and STZ groups as well as increased small β-cell (0 - 2500 μm^2) formation ($p < 0.001$) in the pancreas of the STZ animals. Hearts from Diavite™ treated control and DIO insulin resistant animals presented with smaller infarct sizes ($p < 0.05$) after ischaemia/reperfusion compared to their controls. Diavite™ treatment lead to the increase of basal ($p < 0.01$) and insulin-stimulated ($p < 0.05$) glucose uptake in cardiomyocytes prepared from DIO insulin resistant animals. Diavite™ treatment also led to significantly suppressed PTEN expression and activity ($p < 0.01$) in the DIO insulin resistant animals. In addition, Diavite™ treatment had (i) no obvious detrimental effects in our rat models and (ii) no toxicity over a 3 month period in vervet monkeys.

Conclusion: Our present study has shown that Diavite™ treatment lowers fasting blood glucose levels, stimulates insulin secretion and leads to the formation of β-cells. In addition, oral consumption of Diavite™ elicits cardioprotection against an ischaemic incident. Diavite™ treatment improves insulin sensitivity of cardiomyocytes. Furthermore, it has been established that Diavite™ treatment has no obvious detrimental effects in either of our rat models and no short-term toxic effects over a 3 month period in Vervet monkeys (data not shown).

We thus conclude that in our models, Diavite™ proved safe and it seems as if Diavite™, after short-term use, is beneficial as a dietary supplement.

Opsomming

Inleiding: Vetsug, en die gepaardgaande komplikasies, soos die metaboliese sindroom, hipertensie en kardiovaskulêre siektes, neem wêreldwyd toe. Daar is tans verskeie middels op die mark wat as anti-diabetiese middels geadverteer word. Baie van hierdie geadverteerde produkte het beperkte effektiwiteit en het verskeie newe-effekte. Een so 'n middel, is Diavite™. Diavite™ is 'n plantproduk, wat slegs uit die gedroogte en fyngemaakte peule van die *P. glandulosa* boom bestaan. Hierdie produk word tans bemark as 'n voedselaanvulling met beide bloedglukose en bloeddruk stabiliserende eienskappe, asook die vermoë om glukose gebruik te verbeter. Diavite™ is reeds vrylik beskikbaar van agente sowel as verkrygbaar by verskeie apteke. Die produsente van Diavite™ wil aansoek doen om registrasie vir hul produk by die Medisynebeheerraad (MCC) en hulle vereis daarom wetenskaplike bewyse van die gevolge van die gebruik van hierdie produk.

Doel: Die doel van ons studie was om op versoek van die produksie maatskappy, die doeltreffendheid van Diavite™ *(P. glandulosa)* as 'n anti-diabetiese behandeling te evalueer, sowel as die moontlike meganismes van werking van hierdie plantproduk.

Metodes: Ons het gebruik gemaak van rot modelle van (i) streptozotocin (STZ)-geïnduseerde tipe 1 diabetes en (ii) dieet-geïnduseerde vetsugtig (DIO) insulienweerstandigheid. Manlike Wistar rotte was as (a) tipe 1 diabeties geklassifiseer na 'n eenmalige, intra-peritoneale inspuiting van STZ teen 'n dosis van 40 mg/kg en as (b) insulienweerstandig geklassifiseer, nadat hulle op 'n hoë kalorie dieet (DIO) vir 16 weke was. Die helfte van beide die tipe 1 diabetes en die insulienweerstandige groep diere was met Diavite™ behandel (25 mg/kg/dag) vir 'n tydperk van 4 - 8 weke, afhangende van die model. Die STZ-geïnduseerde tipe 1 diabetes rotte is geslag en die pankreata geoes vir histologiese analise. Diere op die DIO dieet is geslag en (i) die intra-peritoneale vet gewig bepaal, (ii) die geïsoleerde harte blootgestel aan isgemie/herperfusie om die infarkt groottes vas te stel, sowel as die

proteïenuitdrukkingsprofiele te bepaal en (iii) kardiomiosiete was berei om die insulien sensitiwiteit te bepaal. Ten tyde van die slagting is bloedmonsters geneem vir bloedglukose en serum insulien vlak bepaling, vir beide modelle. Additioneel, is 'n standaard toksologie studie met Vervet apies oor 'n 3 maande tydperk uitgevoer.

Resultate: In die model van tipe 1 diabetes (bloed glukose > 10 mmol/L), met 'n β-sel reserwe, is gevind dat Diavite™ behandeling tot verhoogde serum insulien vlakke *(p < 0.001)* in beide kontrole en STZ groepe lei. Diavite™ behandeling lei ook tot ‘n hoër vlak van klein β-sel (0 - 2500 μm^2) vorming *(p < 0.001)* in die pankreas van die STZ diere. Die harte van die Diavite™ behandele kontrole en DIO groep het kleiner infarkt groottes *(p < 0.05)* getoon na isgemie/herperfusie in vergelyking met hul kontrole groepe. Diavite™ behandeling het ook gelei tot verhoogde basal (p < 0. 01) en insulin-gestimuleerde (p < 0. 05) glukose opname in kardiomiosiete wat berei was van DIO insulinweerstandige diere. Diavite™ behandeling het PTEN uitdrukking en aktiwiteit aansienlik onderdruk *(p < 0.01)* in die DIO insulienweerstandige groep diere. Daar is dus gevind dat Diavite™ behandeling (i) geen duidelike nadelige invloed in ons rot-modelle en (ii) geen toksisiteit oor 'n 3 maande tydperk in Vervet apies getoon nie.

Gevolgtrekking: Ons huidige studie toon dus dat Diavite™ behandeling vastende bloedglukosevlakke verlaag, insulien sekresie stimuleer en die proses van β-sell vorming bevorder. Additioneel, is gewys dat wanneer Diavite™ mondelings gebruik word, dit die hart beskerm teen isgemiese insidente. Ons het ook getoon dat Diavite™ behandeling insuliensensitiwiteit van kardiomiosiete verhoog. Verder is daar vasgestel dat Diavite™ behandeling geen ooglopende nadelige gevolge in beide ons rot-modelle getoon het nie en daar geen korttermyn-toksiese effekte oor 'n 3 maande tydperk in Vervet apies (data nie getoon) is nie.

Ons kan dus aflei dat Diavite ™ in ons modelle veilig is en na kort termyn gebruik, voordelig is as 'n dieetaanvulling.

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List of Abbreviations

Units of measurement

AU	Arbitrary units
°C	Degree Celsius
g	Gram
kg	Kilogram
kJ	Kilojoules
L	Litre
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimolar
min	Minutes
µl	Microlitre
%	Percentage
p	Pico
sec	Seconds

Chemicals

2DG	2-deoxy-D-^{3}[H] glucose
BDM	Butanedione monoxime
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
$CuSO_4$	Copper sulfate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
H_2O	Water
HCl	Hydrochloric acid
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
$MgSO_4$	Magnesium sulfate
Na^{+}	Sodium

Na_2CO_3	Disodium carbonate
Na_2HPO_4	Sodium Phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
NaK^+ tartrate	Sodium potassium tartrate
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NADH	Nicotinamide adenine dinucleotide
$NADPH_{OX}$	Nicotinamide adenine dinucleotide phosphate oxidase
Na_3VO_4	Sodium orthovanadate
O_2	Oxygen
PMSF	Phenylmethyl sulfonyl fluoride
PVDF	Polyvinylidene fluoride
TBS	Tris-buffered saline
TTC	Triphenyltetrazolium chloride

Other abbreviations

α	Alpha
ADP	Adenosine diphosphate
ANT	Adenine nucleotide translocase
APAAP	Alkaline Phosphatase-Anti-Alkaline Phosphatase
AS160	Akt substrate of 160 kDa
ATP	Adenosine triphosphate
β	Beta
BMI	Body Mass Index
cAMP	Cyclic adenosine monophosphate
CAP	c-Cbl-associated protein
CARA	Conservation of Agricultural Resources Act
CK2	Casein kinase 2
CPT-1	Carnitine palmitoyl transferase
CyP-D	Cyclophilin D
DCM	Diabetic cardiomyopathy
DIO	Diet-induced-obesity

ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
FADH	Flavin adenine dinucleotide
FATP	Fatty acid transporter
FFA	Free fatty acid
G-6-P	Glucose-6-phosphate
GAP	GTPase-activating protein
GLP-1	Glucagon-like peptide 1
GLUT1/4	Glucose transporter 1/4
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HDL	High-density lipoproteins
HR	Heart rate
IFS	Infarct size
IHD	Ischaemic heart disease
IL-6	Interleuken-6
IPGTT	Intraperitoneal glucose tolerance test
IR	Insulin receptor
IRS	Insulin receptor substrate
λ	Lamda
LDL	Low-density lipoproteins
LVDP	Left ventricular developed pressure
LVH	Left ventricular hypertrophy
MafA	Musculoaponeurotic fibrosarcoma oncogene homolog A
MCC	Medicines Control Council
mPTP	Mitochondrial permeability transition pore
MRC	Medical Research Council
mTOR	Mammalian target of rapamycin
n	Sample number
Ngn3	Neurogenin 3
NHE	Sodium-hydrogen exchanger
NHLBI	National Heart Lung and Blood Institute
NO	Nitric oxide
NOS	Nitric oxide synthase
PAI-1	Plasminogen activator inhibitor-1
PDH	Pyruvate dehydrogenase

PDK-1	Phosphoinositide-dependant kinase 1
Pdx1	Pancreatic and duodenal homeobox 1
PFK-1	Phosphofructokinase-1
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator 1
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP_2 (PtdIns(4,5)P_2)	Phosphatidylinositol (4,5) bisphosphate
PIP_3 (PtdIns(3,4,5)P_3)	Phosphatidylinositol (3,4,5) triphosphate
PKB/Akt	Protein kinase B
PPAR-γ	Peroxisome proliferator-activated receptor γ
PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RIA	Radioimmunoassay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RPP	Rate pressure product
SEM	Standard error of the mean
Ser	Serine
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SH-2	Src homology 2
SHIP	Src homology 2 domain containing inositol 5' phosphatase 2
SR	Sarcoplasmic reticulum
STZ	Streptozotocin
Thr	Threonine
TNF-α	Tumor necrosis factor-α
VDAC	Voltage-dependent anion channel
VLDL	Very low-density lipoproteins
WHO	World Health Organization
ζ	Zeta

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Motivation for research

Being overweight or obese has severe health consequences. The increased incidence of insulin resistance and type 2 diabetes, of which a high percentage can be directly attributed to weight gain [Stein and Colditz, 2004; Eckel *et al.*, 2005], is amongst the most devastating. Type 2 diabetes is a disease of serious concern, due to its associated complications and the enormous impact it has on health care costs. In recognition of this, the need for therapeutics is crucial and normalizing blood glucose levels and improving insulin sensitivity needs to be the primary targets of therapies.

With this in mind, untested remedies advertised as anti-diabetic agents are flooding the market. Many of these advertised products have limited efficacy, limited tolerability and significant side-effects and few of the available products adequately address the underlying defects associated with diabetes, such as obesity and/or insulin resistance. One such remedy, claiming to have anti-diabetic properties, is Diavite^{TM}. Diavite^{TM}, marked by a South African company by the name of Conbrio Brands (Pty) Ltd, is a herbal product that is currently marketed as a food supplement with blood glucose and blood pressure stabilizing properties as well as having the ability to improve glucose homeostasis. It is already freely available from agents as well as sold over the counter at pharmacies at a recommended dose of 7g daily. The producers of Diavite^{TM} are, however, now seeking registration for their product from the Medicines Control Council (MCC) and therefore require solid scientific evidence of its effects.

Diavite^{TM} consists solely of the dried and ground pods of the *P. glandulosa* tree (more commonly known as the Honey mesquite tree) which is part of the Fabaceae (or legume) family. In the past, the pods of this tree were used as the primary foodstuff for the residents of the south-western regions of the North American deserts [Simpson, 1977] and these trees are still widely distributed across a large portion of the south-western United States [Washburn *et al.*, 2002].

Grinding of the plant is thought to improve its use as animal feed. It has, however, been found that the ingestion of the young leaves, pods or beans of the *P. glandulosa* can cause toxicosis if it comprises the majority of the diet of the animals [Washburn *et al.*, 2002]. It appears that sheep are more resistant to the plant's toxic effects and they are thus able to consume a higher percentage of the young leaves, pods and/or beans in their diet than are cattle and goats [Washburn *et al.*, 2002]. The clinical signs of the toxic effects of this plant in animal models include weight loss, ptyalism (drooling), mandibular tremors, tongue protrusion, dysphagia (difficulty in swallowing) and episodes of hypoglycaemia [Washburn *et al.*, 2002].

In South Africa, it was once one of the most common trees found in the dry north-western regions. Beginning in the 1880's numerous *Prosopis* species, including *P. glandulosa*, were introduced to South Africa from various sources in the Americas and became a common ornamental tree in many towns. For many years it was perceived to be a valuable source of shade, animal feed and fuel wood and this was the main reasons that *Prosopis* was introduced from the Americas to many parts of the world. However, in the 1960's this perception changed when the first alarming infestations appeared. During this time, hybridization between two dominant *Prosopis* species namely, *P. velutina* and *P. glandulosa,* started to occur and displayed what is known as "hybrid vigour". These hybrids proved to be very invasive and these hybrid trees lost most of their valuable properties [Steenkamp and Chown, 1996]. In 1983, *P. velutina* and *P. glandulosa* (including their hybrids), where declared category 2 invaders under the Conservation of Agricultural Resources Act of 1983 (Act No. 43 of 1983) (CARA) [Steenkamp and Chown, 1996]. Category 2 invader plants are plants with the proven potential of becoming invasive, but which nevertheless have certain beneficial properties that warrant their continued presence in certain circumstances. By demarcating and controlling the area in which these trees grow according to set regulations, producers can benefit from its resources.

In a pilot study conducted in 2006, we found that genetically type 2 diabetic rats, (Zucker *fa/fa*) treated with Diavite^{TM}, had decreased fasting glucose

levels and an improved intraperitoneal glucose tolerance test (IPGTT) in comparison to untreated controls. In addition to that, cardiomyocytes prepared from these rats, were more insulin sensitive after Diavite^{TM} treatment.

Our aim was therefore, on request of the producing company, to determine the efficacy of Diavite^{TM} (*P. glandulosa*) as an anti-diabetic agent and possible mechanisms of action of this plant product in both type 1 and type 2 diabetic rats. We utilized rat models of streptozotocin-induced type 1 diabetes and diet-induced obese (DIO) insulin resistance.

Disclosure of Interest

We hereby declare, as per contractual agreement between the University of Stellenbosch and Dormell Properties 528 (Pty) Ltd (Registration number: 2005/031723/07), the company licensing Conbrio Brands (Pty) ltd to distribute Diavite^{TM}, that there was no personal financial gain for the researchers in this work. The researchers only retained the intellectual information that they generated through their studies and the right to publish these findings in peer reviewed scientific journals of their choice.

We acknowledge the grant money from Dormell Properties to partially fund the work as well as a THRIP grant from the NRF in a 1:1 ratio to complement this.

Signed on the …… day of ……. 2010 at…….

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(Prof B Huisamen) (Me C Hill)

CHAPTER 1

Literature review

1. THE "PANDEMIC" OF OBESITY

The prevalence of obesity has focused attention on a worldwide problem that is on a rampant increase. Obesity is a serious public health issue, escalating in countries with low and middle income [World Health Organization, 2006]. It has been estimated that there are approximately 1.1 billion overweight (body mass index: BMI ≥ 25 kg/m^2) adults worldwide and a further 312 million adults that are obese (BMI ≥ 30 kg/m^2) [Hossain *et al.*, 2007; Deitel, 2003] with South Africa also not being spared in this global increase [Puoane *et al.*, 2002].

Over the past decades, much research has gone into the pathophysiology of obesity and its associated complications. According to the National Heart Lung and Blood Institute (NHLBI), obesity is one of the leading causes of preventable deaths globally [National Heart Lung and Blood Institute, 1998]. This is due to the strong association between obesity and the risk of developing metabolic abnormalities such as insulin resistance, type 2 diabetes and hypertension. All of which is well-documented risk factors for the development of cardiovascular diseases [Stein and Colditz, 2004; Smith, 2007; Eckel *et al.*, 2005].

The sections that follow will discuss the link between obesity and disease states, such as insulin resistance, type 2 diabetes and cardiovascular disease and why research into obesity and its associated complications and the need for therapies, are so important.

2. PHYSIOLOGICAL MECHANISMS OF INSULIN ACTION

Before discussing the link between obesity and insulin resistance, it is appropriate to give a brief discussion on the physiological importance and the mechanisms of action of insulin under normal physiological conditions.

2.1 Overview of insulin action

All animals undergo a cycle of feeding and starvation. During periods of feeding, nutrients are stored in forms that can be used as energy sources during later periods of fasting. Insulin, which is a hormone produced by the β-cells of the pancreas, regulates this process. In response to hyperglycaemia the β-cells secretes insulin, which in turn stimulates the uptake of glucose by peripheral insulin sensitive tissues.

Glucose homeostasis is most affected by skeletal muscle, liver and adipose tissue. In skeletal muscle and liver, insulin stimulation is responsible for the synthesis of glycogen from glucose and it is responsible for the inhibition of glycogenolysis. In the liver, insulin reduces gluconeogenesis, which in turn prevents an influx of additional glucose into the circulation [Saltiel and Kahn, 2001]. In adipose tissue, insulin action inhibits lipolysis and stimulates glucose uptake into the tissue. The cumulative effect of all these changes is to enhance glucose utilization, reduce circulating glucose levels and increase the conversion of glucose into glycogen or fat for storage [Opie, 1998; Kim *et al.*, 2006] and thus keep the homeostatic balance.

Section 2.2 on page 3 describes the involvement of insulin in glucose uptake and storage and the proteins and mechanisms responsible for insulin signaling termination. It is important to note that, although the pathways described below are illustrated in a linear fashion, it should not be forgotten that each pathway could, under certain circumstances, activate others.

2.2 From insulin stimulation to glucose storage

Circulating insulin reaches its target tissue, where it interacts with its associated receptor, the insulin receptor (IR) [Rosen, 1989; Ebina *et al.*, 1985; Ullrich *et al.*, 1985]. The IR belongs to a large subfamily of receptor tyrosine kinases [Patti and Kahn, 1998], consisting of two extracellular α- and two transmembrane β-subunits. This subfamily of receptors are disulphide-bonded proteins that function as allosteric enzymes, in which the binding of insulin to the α-subunit induces a rapid conformational change, resulting in the autophosphorylation of specific tyrosine residues of the intracellular region of the β-subunit through a transphosphorylation mechanism [Van Obberghen *et al.*, 2001; Hubbard, 1997]. These residues are recognized by phosphotyrosine-binding (PTB) domains of adaptor proteins such as members of the insulin receptor-substrate (IRS) family [Saltiel and Kahn, 2001; Lizcano and Alessi, 2002]. Autophosphorylation results in the activation of the tyrosine kinase activity of the receptor [Lee *et al.*, 1997]. The activated IR phosphorylates substrate proteins on tyrosine residues, where these phosphorylated tyrosine residues act as “docking sites” for downstream effectors that contain Src homology 2 (SH2) domains. Many of these SH2-containing proteins are adaptor molecules, such as the p85 regulatory subunit of phosphoinositide 3-kinases (PI3K). The metabolic response to insulin is primarily mediated via the PI3K/PKB/Akt pathway, which is a main prosurvival pathway. The catalytic subunit of PI3K, p110, then phosphorylates phosphatidylinositol (4,5) bisphosphate (or Ptdlns(4,5)P_2, abbreviated as PIP_2) leading to the formation of phosphorylated phosphatidylinositol (3,4,5) triphosphate (or Ptdlns(3,4,5)P_3, abbreviated as PIP_3). This process markedly stimulates the activation of serine kinase Akt (also known as protein kinase B, abbreviated as PKB or PKB/Akt) which is recruited to the plasma membrane. This incident predominantly relies on the phosphorylation of PKB/Akt at the Thr^{308} and Ser^{473} sites, by phosphoinositide-dependent kinase 1 (PDK-1) and mammalian target of rapamycin/rapamycin intensive companion of mTOR (mTOR/Rictor) complex respectively [Alessi *et al.*, 1997; Hresko and Mueckler, 2005]. Once PKB/Akt has been activated, it enters the cytoplasm where it leads to the phosphorylation and inactivation of glycogen synthase

kinase 3 (GSK-3). The inactivation of GSK-3, in turn, promotes glucose storage as glycogen. Figure 1.1 (page 6), is a simplified overview of insulin stimulated GLUT4 translocation and glucose uptake.

2.3 From insulin stimulation to GLUT4 translocation

Glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) are the two main glucose transporters present in cardiomyocytes [Abel, 2004]. GLUT4, which continuously cycles from intracellular stores to the plasma membrane, plays an important role in glucose homeostasis in both animals and humans. At basal levels, GLUT4 resides in intracellular, cytoplasmic vesicles, however, during insulin stimulation GLUT4 translocates to the plasma membrane where it transports postprandial glucose from the extracellular environment into cells [Huang and Czech, 2007; Wallberg-Henriksson and Zierath, 2001]. Insulin is thus the hormone responsible for increasing glucose transport by increasing the rate of GLUT4-vesicle exocytosis and by slightly decreasing the rate of internalization [Pessin *et al.*, 1999].

In conjunction with insulin stimulated translocation, increased work demand and ischaemia [Stanley *et al.*, 1997 (a); Young *et al.*, 1997] also lead to GLUT4 translocation. The translocation of GLUT4 takes place by means of both a PI3K-dependent pathway and a PI3K-independent pathway [Pessin *et al.*, 1999; Lizcano and Alessi, 2002].

2.3.1 PI3K-dependent pathway

Insulin-stimulated GLUT4 translocation from vesicular intracellular compartments to the cell surface is a multiple-step process that involves intracellular sorting, vesicular transport to the cell surface along cytoskeletal elements, docking, priming and fusion of the GLUT4 storage vesicles with the cell surface.

PKB/Akt, activated via PI3K, phosphorylates the downstream protein, Akt substrate of 160 kDa (AS160, originally known as TBC1D4) and TBC1D1 at Thr^{642} and Thr^{596}, respectively [Kahn *et al.*, 2002; Sano *et al.*, 2007]. This enhances binding of the scaffolding protein 14-3-3 to these proteins, which is thought to inhibit Rab-GTPase-activating protein (GAP) activity towards particular Rab isoform(s). Inhibition of GAP promotes conversion of less active GDP-loaded Rab to more active GTP-loaded Rab [Sakamoto and Holman, 2008]. The more active GTP-loaded Rab then allows GLUT4 storage vesicles to move to and fuse with the plasma membrane [Sano *et al.*, 2007; Sakamoto and Holman, 2008] (refer to Figure 1.1).

2.3.2 PI3K-independent pathway

It has also been suggested that a second pathway, a PI3K-independent pathway occurs as a consequence of Cbl tyrosine phosphorylation [Ribon and Saltiel, 1997; Liu *et al.*, 2003]. The protooncogene, Cbl and the adaptor protein c-Cbl-associated protein (CAP) [Ribon and Saltiel, 1997; Ribon *et al.*, 1998] are recruited to the IR by adaptor molecules containing PH and SH2 domains [Liu *et al.*, 2003]. Upon phosphorylation, the Cbl-CAP complex translocates to lipid raft domains in the plasma membrane. The phosphorylated Cbl then recruits the adaptor protein CrkII, which forms a constitutive complex with the Rho-family guanyl nucleotide-exchange protein, C3G, to the lipid raft [Chiang *et al.*, 2001]. Once translocated, C3G comes into close proximity with the G protein TC10 [Chiang *et al.*, 2001], which promotes GLUT4 translocation to the plasma membrane [Huang and Czech, 2007; Wallberg-Henriksson and Zierath 2001; Watson and Pessin, 2006; Bryant *et al.*, 2002]. Once brought into the proximity of fusion machinery at the plasma membrane by the cytoskeleton, the GLUT4 vesicle can dock and subsequently fuse, exposing GLUT4 to the extracellular environment [Saltiel and Kahn, 2001] (refer to Figure 1.1). It is important to note that the cytoskeleton is crucial in maintaining glucose homeostasis as it is involved in the distribution of IRS [Clark *et al.*, 2000; Chang *et al.*, 2004], the translocation of GLUT4 to the membrane [Tsakiridis *et al.*, 1994; Vollenweider *et al.*, 1997;

Wang *et al.*, 1998] and the internalization of the IR [Boura-Halfon *et al.*, 2003; Shorten *et al.*, 2007]. Thus, dysfunction of the cytoskeleton may lead to insulin resistance.

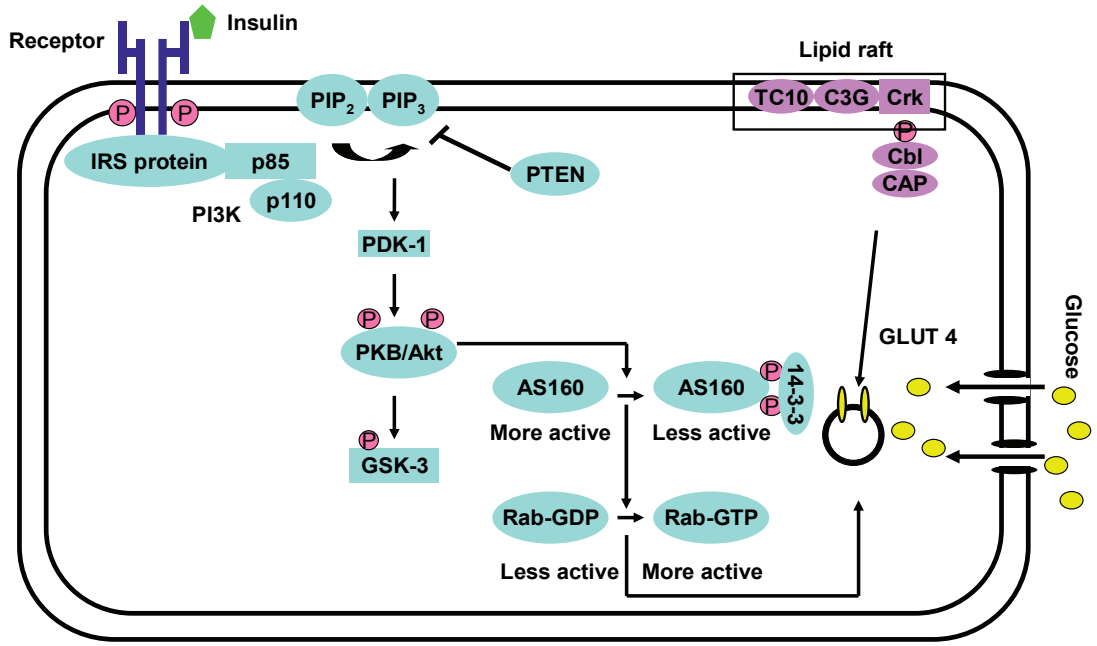


Figure 1.1: Simplified overview of insulin stimulated GLUT4 translocation and glucose uptake. The IR undergoes autophosphorylation and catalyses the phosphorylation of members of the IRS family. Upon tyrosine phosphorylation, IRS proteins interact with signaling molecules through their SH2 domains, resulting in activation of PI3K and downstream PIP_3-dependent protein kinase, PDK-1. PDK-1 phosphorylates and activates PKB/Akt, which enters the cytoplasm and leads to the phosphorylation and inactivation of GSK-3. Inactivated GSK-3 promotes glucose storage as glycogen. Activated PKB/Akt also phosphorylates downstream protein, AS160, which enhances binding of scaffolding protein 14-3-3. This binding inhibits GAP, promoting the conversion of active GDP-loaded Rab to active GTP-loaded Rab. This allows for GLUT4 vesicle translocation to the cell surface. These pathways act in a concerted fashion to coordinate the regulation of vesicle trafficking, protein synthesis and gene expression. Adapted from Saltiel and Kahn (2001) and Watson and Pessin, (2006). IRS: insulin receptor substrate; PI3K: phosphoinositide 3-kinase; PIP_2: phosphatidylinositol (4,5) bisphosphate; PIP_3: phosphatidylinositol (3,4,5) triphosphate; PTEN: phosphatase and tensin homolog deleted on chromosome 10; PDK-1: phosphoinositide-dependent kinase 1; PKB/Akt: protein kinase B; GSK-3: glycogen synthase kinase 3; GLUT4: glucose transporter 4; CAP: Cbl-associated protein

2.4 Negative regulators and process of insulin signaling termination

The lipid phosphatases, phosphatase and tensin homolog deleted on chromosome ten (PTEN) and Src homology 2 domain containing inositol 5' phosphatase 2 (SHIP2) have been implicated as negative regulators of the PI3K pathway [Sasaoka *et al.*, 2006; Clement *et al.*, 2001]. PTEN and SHIP2 dephosphorylate the PI3K product PIP_3 (which is a lipid second messenger), thereby blocking the activation of PKB/Akt and the downstream substrates. Hence, PTEN is thought to be the main downregulator of this survival pathway [Hlobilkova *et al.*, 2003; Mocanu and Yellon, 2007].

PTEN is ubiquitously present in normal cells and its degree of activity depends on its intracellular concentration. However, its activity can be downregulated by phosphorylation or oxidation. The main enzyme responsible for the phosphorylation of PTEN is considered to be casein kinase 2 (CK2) [Torres and Pulido, 2001], but other kinases have been identified that might also be able to phosphorylate PTEN, at least in an *in vitro* model [Mehenni *et al.*, 2005]. The opinion is that in its phosphorylated state, PTEN is inactive and as such is more stable against proteasomal degradation [Vazquez *et al.*, 2000]. PTEN can also be inactivated through oxidation induced by free radicals (ROS) [Leslie *et al.*, 2003]. This seems to be the main process regulating PTEN activity in the acute setting. Recent documented research suggests that insulin, which is known to activate the PI3K/PKB/Akt pathway, may induce the activation of nicotinamide adenine dinucleotide phosphate oxidase ($NADPH_{OX}$) which in turn releases ROS [Seo *et al.*, 2005; Espinosa *et al.*, 2009]. This ROS could be responsible for the inhibition of PTEN [Seo *et al.*, 2005], which would then be followed by PKB/Akt activation due to PIP_3 accumulation. Furthermore, it has been demonstrated that hydrogen peroxide produced at the mitochondrial level can also inhibit PTEN [Conner *et al.*, 2005].

Amongst the factors that induce transcription of PTEN are the peroxisome proliferator-activated receptor γ-agonists (PPAR-γ) [Teresi *et al.*, 2006] and the tumor suppressor p53 [Wang *et al.*, 2005]. The regulation of PTEN and its activity is complex and is yet not completely understood, but from what is

known, PTEN seems to be an important “switch” in the aim of maintaining cellular homeostasis and normal development.

SHIP is the other lipid phosphatase responsible for insulin signaling termination. SHIP-1 has a restricted hematopoietic expression and SHIP-2 is ubiquitously expressed [Pesesse *et al.*, 1997; Bruyns *et al.*, 1999] in skeletal muscle, heart, placenta and the pancreas and to a lesser extent in the liver and kidney [Pesesse *et al.*, 1997; Vollenweider *et al.*, 1999]. The exact mechanism of action of SHIP-2, especially in the heart, is still unclear. However, over-expression of SHIP-2 in β-cells was shown to inhibit PKB/Akt activity and SHIP-null cells exhibit prolonged activation of PKB/Akt upon stimulation [Aman *et al.*, 1998]. Research also shows that homozygous mice lacking SHIP-2 develop severe neonatal hypoglycaemia or prenatal death and adult SHIP-2 heterozygous mutant mice demonstrate insulin sensitivity, which is associated with increased translocation of GLUT4 to the plasma membrane in response to insulin treatment [Clement *et al.*, 2001].

In addition, it is worth mentioning that both phosphatases are reported to have a negative impact on pathological conditions associated with obesity, such as insulin resistance [Wejesekara *et al.*, 2005; Sasaoka *et al.*, 2005] and diabetes [Sasaoka *et al.*, 2006; Mocanu and Yellon, 2007]. The activity and/or the expression of these phosphatases have been reported to be elevated in skeletal muscle and fat tissue under conditions of insulin resistance [Sasaoka *et al.*, 2005; Lo *et al.*, 2007]. Therefore blocking PTEN [Oudit *et al.*, 2004] or SHIP-2 may prove important, particularly in increasing myocardial survival following an ischaemic incident.

3. THE LINK BETWEEN OBESITY AND INSULIN RESISTANCE

Insulin resistance is defined as a reduced responsiveness of a target cell or a whole organism to the insulin concentration to which it is exposed [Shanik *et al.*, 2008]. The influence of body fat on insulin action is very important and the relation between obesity, especially when it is centrally located [Kissebah *et al.*, 1989], insulin resistance and the risk for developing type 2 diabetes is well recognized.

There are two general mechanisms that have been identified as links between obesity and insulin resistance and they are (i) the role of fatty acids and (ii) the role of adipokines and pro-inflammatory cytokines. Figure 1.2 (page 14) is a simplified schematic representation of the inter-play between the factors thought to be involved in the development of insulin resistance.

3.1 The fatty acid hypothesis

The major contributor to the development of insulin resistance is an overabundance of circulating fatty acids in overweight and obese individuals. Elevated levels of free fatty acids reduce glucose uptake by the heart [Boden *et al.*, 1994] by increasing muscular and myocardial free fatty acid (FFA) uptake and oxidation [Barsotti *et al.*, 2009]. FFA's are mainly derived through the lipolysis of triglyceride-rich lipoproteins from adipose tissue, by the action of the cyclic AMP-dependent enzyme lipoprotein lipase [Eckel, 1989, Eckel *et al.*, 2005]. During the development of insulin resistance, there is an increased amount of lipolysis, which produces more fatty acids. This in turn inhibits the antilipolytic effect of insulin, creating additional lipolysis. At cellular level, fatty acids and their metabolic products can create insulin resistance by added substrate availability and by modifying downstream signaling. Thus in short: circulating FFA's increase hepatic glucose production and diminish inhibition of glucose production by insulin, while inhibiting glucose utilization by peripheral tissues. A detailed description of fatty acid metabolism follows in section 5.3.1 on page 28.

3.2 Adipokines and pro-inflammatory cytokines as contributors to insulin resistance

A second, multifaceted mechanism linking obesity to insulin resistance is the inflammatory hypothesis. In this section, we will review the effect that (i) adipokines, namely leptin and adiponectin and (ii) the pro-inflammatory cytokines, secreted by the adipose tissue, have on the development of insulin resistance.

3.2.1 Adipokines

Leptin is an adipokine secreted by the adipocytes and has a vital role in energy homeostasis [Pittas *et al.*, 2004]. Although the main target of leptin is the appetite centre in the brain, it seems to have effects on insulin action in peripheral tissues, as well as on blood vessels and pancreatic β-cells [Crowley, 2008; Ronti *et al.,* 2006; Seufert, 2004].

Insight into the relationship between leptin and insulin resistance, comes from studies of leptin deficiency syndromes. Leptin deficient mice (*ob/ob*) exhibit hyperphagia, obesity, hypercortisolemia, infertility and diabetes [Zhang *et al.*, 1994]. However, once exogenous leptin is administered, these abnormalities are reversed [Pelleymounter *et al.,* 1995].

In obese individuals leptin levels are normally very high and more than sufficient to suppress the appetite and increase the metabolism. This, however, does not happen and it is believed that obesity may be the result of a resistance to leptin [Mark *et al.*, 2002]. It is thought that hyperinsulinaemia promotes both insulin resistance and stimulation of leptin production and secretion from adipose tissue. This may in turn enhance leptin resistance by further desensitizing its signal transduction pathways [Seufert, 2004].

Adiponectin is another anti-inflammatory cytokine that has been shown to both improve insulin sensitivity and inhibit many steps in the inflammatory process [Nawrocki *et al.*, 2004]. In the liver, adiponectin regulates cells to decrease gluconeogenesis [Sheng and Yang, 2008; Combs *et al.*, 2001] and to increase fatty acid oxidation. In skeletal muscle, adiponectin increases glucose transport and uptake and enhances fatty acid oxidation [Xu *et al.*, 2003].

Unlike most adipose tissue products, adiponectin is negatively related to fat mass [Kiortsis *et al.*, 2005]. This is observed in studies done on obese human patiens and in obese rodent models. Obese patients are found to have lower baseline levels of adiponectin compared to healthy individuals [Hotta *et al.*, 2000; Beltowski, 2003; Matsuzawa *et al.*, 2004]. In obese and type 2 diabetes rodent models, insulin resistance is reversed after administration of adiponectin [Yamauchi *et al.*, 2004]. Although, obesity is associated with low levels of adiponectin, the negative correlation between adiponectin and insulin sensitivity is not dependent on adipose tissue alone. Numerous studies report that adiponectin levels decrease with increasing BMI, plasma glucose, insulin and triglycerides, in rodents and humans [Rajala and Scherer, 2003]

3.2.2 Pro-inflammatory cytokines

Tumor necrosis factor-α (TNF-α) is one of the primary pro-inflammatory cytokines that has been implicated in the pathogenesis of insulin resistance. Hotamisligil was one of the first researchers to propose the notion that obesity is a low-grade inflammatory state [Hotamisligil *et al.*, 1993]. In this paper, Hotamisligil and colleagues (1993) documented that adipose tissue expressed TNF-α and that its expression was elevated in the obese state. They proposed that the increased expression of TNF-α could contribute to insulin resistance. Subsequent studies have largely supported this hypothesis, such as studies done by Moller (2000) on TNF-α-null and TNF-α receptor-null mice and Fried and Halaas (1998), where they too observed an enhanced TNF-α production in adipose tissue obtained from obese and insulin resistant rodents and human subjects.

In addition, when adipose tissue of obese rodents was compared to their lean counterparts, increased levels of multiple pro-inflammatory cytokine gene expression were observed [Weisberg *et al.*, 2003; Xu *et al.*, 2003; Kern *et al.*, 2001] and weight loss reduced the TNF-α levels. Because the above-mentioned effects appear before the development of insulin resistance and during high-fat feeding, it further supports the belief that adipose-derived inflammatory factors may have a causal role in the development of high-fat diet-induced insulin resistance.

Various mechanisms have been proposed to explain the effect of TNF-α on the development of obesity-related insulin resistance. Possible mechanisms include increased release of FFA by adipocytes through additional lipolysis of adipose tissue triglyceride stores [Eckel *et al.*, 2005], reduction in adiponectin synthesis [Bruun *et al.*, 2003] and impairment of insulin signaling [Greenberg and McDaniel, 2002; Hotamisligil and Spiegelman, 1994].

Interleukin-6 (IL-6) is a circulating cytokine, which is normally secreted by activated macrophages and lymphocytes. However, in non-acute inflammatory conditions its major source is adipose tissue [Mohamed-Ali *et al.*, 1997]. IL-6 is a pleiotropic cytokine (cytokine affecting the activity of multiple cell types) and its effects range from inflammation to tissue injury [Papanicolaou *et al.*, 1998]. For this review, we will only be focusing on its link to insulin resistance.

The link between IL-6 and insulin resistance is supported by epidemiological studies as well as genetic studies [Klover *et al.*, 2003; Hebert *et al.*, 2006; Qi *et al.*, 2006]. There is a positive correlation between plasma IL-6 levels and human obesity and insulin resistance [Vozarova *et al.*, 2001, Kern *et al.*, 2001; Pradhan *et al.*, 2001; Papanicolaou *et al.*, 1998]. As with TNF-α, IL-6 is thought to exert its adverse effects by increasing circulating FFA with its well described adverse effects on insulin sensitivity [Boden and Shulman, 2002], enhancing hepatic glucose production and decreasing adiponectin secretion [Fasshauer *et al.*, 2003]. Weight loss is found to significantly reduce IL-6

levels in both adipose tissue and serum [Bastard *et al.*, 2000]. In the clinical set-up, elevated levels of IL-6 are used as a predictor of type 2 diabetes development [Pradhan *et al.*, 2001] and risk of future myocardial infarction [Ridker *et al.*, 2000].

In addition to the FFA hypothesis and the involvement of adipokines and pro-inflammatory cytokines, growing evidence links plasminogen activator inhibitor-1 (PAI-1) with obesity and insulin resistance. Adipose tissue has been found to be a key source of PAI-1, with the bulk production in visceral adipose tissue [He *et al.*, 2003]. PAI-1 is a key regulatory protein in processes such as tissue fibrinolysis, cell migration, angiogenesis and tissue remodeling [Lijnen, 2005]. It has been found that PAI-1 deficient mice have reduced adiposity and an improved metabolic profile [Schafer *et al.*, 2001] and PAI-1 deficiency attenuated diet-induced obesity and insulin resistance in C57BL/6 mice [De Taeye *et al.*, 2006]. Furthermore, in mouse models, the absence or inhibition of PAI-1 through genetic alteration in adipocytes protects against insulin resistance by promoting glucose uptake and adipocyte differentiation via increased PPAR-α expression [Liang *et al.*, 2006].

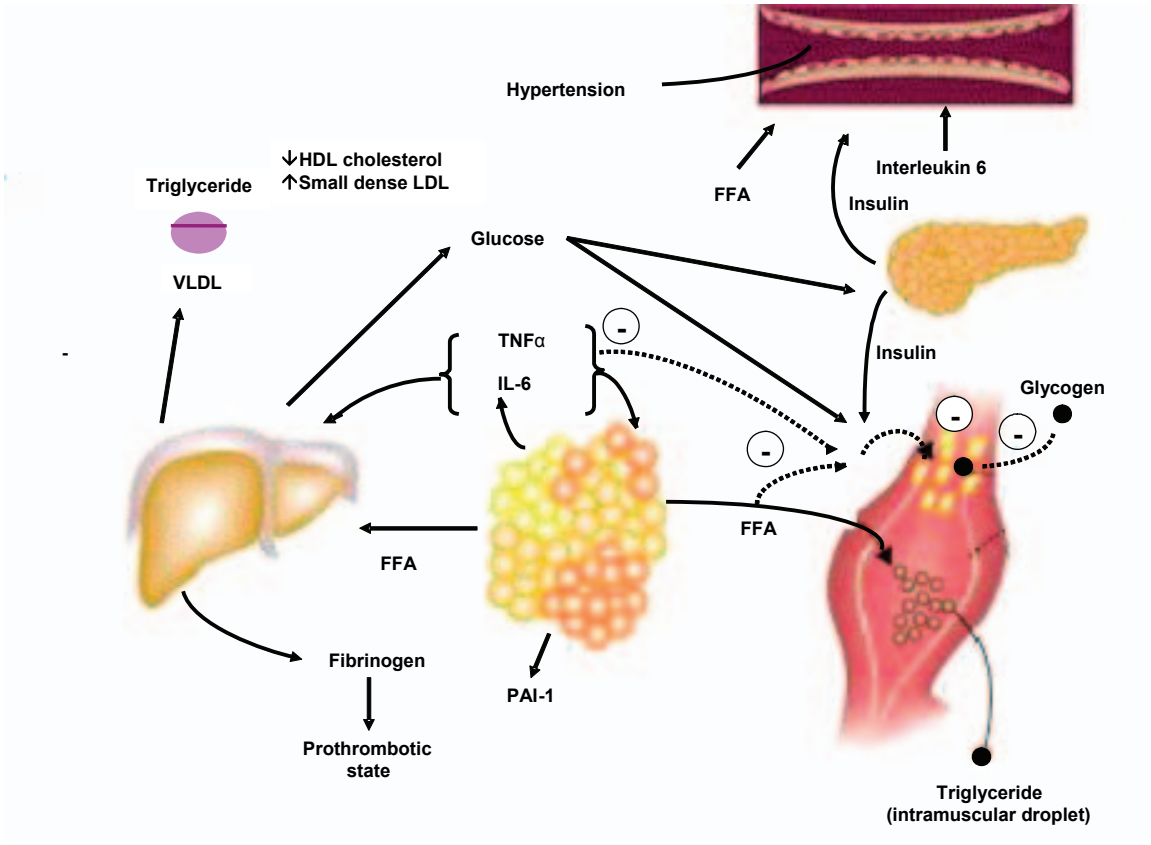


Figure 1.2: Simplified, schematic representation of the inter-play between factors involved in the development of insulin resistance. FFA's are released in abundance from an expanded adipose tissue mass. In the liver, FFA's lead to an increased production of glucose, triglycerides and secretion of VLDL. Associated abnormalities include reductions in HDL cholesterol and an increased density of LDL. FFA's also reduce insulin sensitivity in muscle by inhibiting insulin-mediated glucose uptake. Associated defects include a reduction in glucose conversion to glycogen and increased lipid accumulation in triglyceride. Increases in circulating glucose and FFA increase pancreatic insulin secretion resulting in hyperinsulinemia. Contributory to the insulin resistance produced by excessive FFA is the paracrine and endocrine effect of the proinflammatory state. The enhanced secretion of IL-6 and TNF-α among others results in more insulin resistance and lipolysis of adipose tissue triglyceride stores to circulating FFA. The increased IL-6 may enhance hepatic glucose production, the production of VLDL by the liver and insulin resistance in muscle. Cytokines and FFA's also increase the production of PAI-1 by the liver that complements the overproduction of PAI-1 by adipose tissue. This results in a pro-thrombotic state. Adapted from Eckel *et al.,* (2005). VLDL: very low-density lipoproteins; HDL: high-density lipoproteins; LDL: low-density lipoproteins; FFA: free fatty acids; PAI-1: plasminogen activator inhibitor-1; TNFα: tumour necrosis factor-α

4. THE LINK BETWEEN INSULIN RESISTANCE AND DIABETES

Insulin resistance is the earliest detectable clinical manifestation in the development of diabetes. The section below describes this link.

4.1 Diabetes mellitus

Wild *et al.* (2004) estimated, based on data obtained from the World Health Organization, that there were approximately 171 million individuals with diabetes mellitus at the start of 2000 and that this number would increase to 366 million by 2030.

Diabetes mellitus is a disorder in the metabolism of blood glucose. It occurs when the body's production of insulin is inadequate or when the body no longer responds effectively to endogenous insulin. Both these abnormal responses lead to chronic hyperglycaemia, accompanied by abnormal metabolism of carbohydrate, fat and protein. Diabetes can be a life threatening condition if not detected early and continually monitored. According to WHO calculations, almost 3 million deaths annually are as a result of diabetes [Wild *et al.*, 2004]. Diabetes mellitus is classified into four major groups: 1) type 1 diabetes, caused by the destruction of pancreatic β-cells and thus characterized by the inability of the β-cell's to synthesize insulin [Daneman, 2006]; 2) type 2 diabetes, characterized by insulin resistance and β-cell dysfunction, whereby the body becomes resistant to the effects of insulin, presumably due to defects in the insulin signaling pathway [Olefsky *et al.*, 1982; DeFronzo *et al.*, 1979; Turner *et al.*, 1979]; 3) other types of diabetes, including genetic diabetic forms [Winter, 2003; Simonen *et al.*, 2006], pancreas disorders and endocrinopathies [Resmini *et al.*, 2009]; and 4) gestational diabetes, with diabetes onset during pregnancy [Serlin and Lash, 2009; Alwan *et al.*, 2009].

For the purpose of this review, only type 1 and type 2 diabetes will be discussed in further detail.

4.1.1 Type 1 diabetes mellitus

Type 1 diabetes accounts for about 5 to 10% of all cases of diabetes [Daneman, 2006]. Because the manifestation of type 1 diabetes was thought to only develop in childhood or adolescence this form of diabetes was formerly termed juvenile-onset diabetes or insulin-dependent diabetes mellitus. However, it is now recognized that type 1 diabetes can occur at any age [Seissler, 2008].

It is widely accepted that type 1 diabetes results from a chronic autoimmune response directed against the insulin-producing β-cells in the islets of Langerhans. The destruction may occur over months or even years and can eventually result in complete loss of endogenous insulin supply. The autoimmune trigger against the β-cells is most likely caused by environmental agent(s) in conjunction with a predisposing genetic background. The mechanisms of β-cell damage in type 1 diabetes is not yet completely understood, but it is thought to involve mechanisms such as Fas/FasL, perforin/granzyme, reactive oxygen and nitrogen species and pro-inflammatory cytokines [Yoon and Jun, 2005; Cnop *et al.*, 2005; Eizirik and Mandrup - Poulsen, 2001]. Binding of these cytokines to their receptors on the β-cells activates MAP-kinases and the transcription factors STAT-1 and NFkappa-β. This is known to bring about functional impairment, endoplasmic reticulum stress and ultimately apoptosis [Eizirik and Mandrup - Poulsen, 2001; Eizirik *et al.*, 2008].

This response of β-cell destruction ultimately leads to insulin deficiency that requires hormone replacement therapy to restore physiological control of blood glucose [Daneman, 2006]. Patients thus require lifelong insulin for survival as there is no cure for this immune-mediated disease.

Whole pancreas transplantation [Bertuzzi *et al.*, 2006], pancreatic islet transplantation [Hakim, 2006] and gene therapy approaches have all been used in the aim to prevent the morbidity and mortality associated with this complex disease [Bagley *et al.*, 2008]. Recently, stem cell transplantation [Ramiya *et al.*, 2000; Seaberg *et al.*, 2004; Suzuki *et al.*, 2004], β-cell

replication [Bouwens and Rooman, 2005], reprogramming of mature β-cells [Bonner-Weir *et al.*, 2000 (b), 2004; Gao *et al.*, 2003] and *in vivo* regeneration of β-cells [Dor *et al.*, 2004], have also been tested as possible therapies, with promising results.

There are reported cases of individuals that have, at the time of diagnosis, a residual β-cell function sufficient to prevent the development of acute metabolic ketosis [Seissler, 2008]. This late stage of clinical presentation and the absence of immediate insulin requirement often lead to a misclassification as type 2 diabetes. Autoimmune destruction of the β-cells of the pancreas is more than 90% complete by the time hyperglycaemia becomes clinically evident in individuals with type 1 diabetes mellitus [Ryu *et al.*, 2001]. Thus, drug treatment aimed at preventing or prolonging the onset of this disease, by prolonging the "window period", would therefore be beneficial in a clinical setup.

4.1.2 Type 2 diabetes mellitus

Type 2 diabetes has a strong polygenic background on which environmental factors act and largely determines the clinical presentation. This disease usually develops in adulthood and is linked to obesity, lack of physical activity and unhealthy diets. Type 2 diabetes is the more common type of diabetes and affects 90 – 95% of patients with diabetes [Moller, 2001]. Although a positive correlation between BMI and risk for type 2 diabetes mellitus was identified in studies such as these two large epidemiologic reports - the Nurses' Health Study [Colitz *et al.*, 1995; *Hu et al.*, 2001] and the Health Professionals' Follow-up Study [Chan *et al.*, 1994], not everyone who is obese has type 2 diabetes mellitus and not everyone who has type 2 diabetes mellitus has an elevated BMI [Colitz *et al.*, 1995; Chan *et al.*, 1994].

4.1.2.1 Pancreatic cell function

In recent years major advances have been made in our understanding of how the pancreas develops [Servitja and Ferrer, 2004; Habener *et al.*, 2005; Collombat *et al.*, 2006]. A normal pancreas contains approximately 1,000,000 islets of Langerhans, in which different types of endocrine cells reside [Marchetti *et al.*, 2008]. Four main cell types exist in the islets, namely, the insulin secreting β-cells, which represent the majority of islet endocrine cells (60 to 80%), followed by glucagon containing α-cells (20 to 30%), somatostatin containing δ-cells (5 to 15%) and pancreatic polypeptide cells [Marchetti *et al.*, 2008]. The α- and β-cells monitor the concentration of glucose in the blood and secrete hormones with opposite effects. Both these cell types play a pivotal role in glucose homeostasis of a healthy human subject, by ensuring that the individuals' plasma glucose concentration remains within the narrow physiological range of 4 to 8 mmol/L (fasting blood glucose level) [Andrews *et al*., 1998]. The amount of insulin released by the β-cells varies according to the nature, administration of the stimulus and the existing glucose concentration [Guillausseau *et al.*, 2008]. The released insulin stimulates the uptake of glucose by cells and its conversion to glycogen in the liver and muscle. In contrast, the islet α-cells secrete glucagon, which is the hormone responsible for stimulating the breakdown of glycogen to glucose in the liver, increasing the blood glucose levels. The β-cells are the cells of interest for this review.

4.1.2.2 β-cell mass

Over the past few decades, it has become clear that the β-cell mass is dynamic, with a significant capacity to adapt to changes in insulin demand. For example, non-diabetic obese individuals have more β-cells than lean individuals or obese individuals with type 2 diabetes [Nielsen *et al.,* 1992]. Optimal control of blood glucose concentrations depends on delicate changes in insulin production and secretion by the pancreatic β-cells. For this reason, it is very important for β-cell mass to be closely regulated.

β-cell mass is determined by the interplay between β-cell mass expansion and reduction mechanisms. Expansion can occur through increases in β-cell hypertrophy, hyperplasia and neogenesis. In contrast, reduction occurs through β-cell atrophy or death by means of apoptosis [Bonner-Weir and Weir, 2005; Dor *et al.*, 2004; Halban, 2004; Marchetti *et al.*, 2008; Rhodes, 2005]. Accordingly, β-cell mass is achieved through the manipulation of any of these mechanisms, alone or in combination.

Under normal conditions, the main process involved in β-cell mass directly after birth, is replication, which is followed by the process of neogenesis [Bonner-Weir, 2001; Marchetti *et al.*, 2008; Rhodes, 2005]. During this phase of development, the rate of apoptosis is low, which allows for cell growth. Throughout childhood and adolescence there is a balance between replication, neogenesis and apoptosis. This results in adequate β-cell mass throughout adulthood. During old age, however, apoptosis increases to a point whereby it outweighs neogenesis and replication, which leads to a β-cell mass decrease [Marchetti *et al.*, 2008; Rhodes, 2005].

During pathological conditions such as insulin resistance, due to obesity, the pancreatic β-cell mass is increased [Prentki and Nolan, 2006]. This increased β-cell mass leads to enhanced insulin secretion (hyperinsulinaemia), to compensate for the increased insulin demand [Weyer *et al.*, 1999; Kahn *et al.*, 1993; Prentki and Nolan, 2006]. However, if hyperinsulinaemia continues the β-cells are unable to fully compensate for the decreased insulin sensitivity and start to deteriorate in function [Kahn, 2001; Perley and Kipnis, 1966; Polonsky *et al.*, 1988]. This leads to β-cell dysfunction and progressive loss [Kahn, 2003; Ferrannini and Mari, 2004; Buchanan, 2003] due to apoptosis and the subsequent development of type 2 diabetes [Buchanan, 2001; Butler *et al.*, 2003].

The apoptosis seen in patients with type 2 diabetes is thought to be because of glucotoxicity or lipotoxicity. Chronic exposure of rodent β-cells to high glucose levels displays alterations in β-cell phenotype, alterations that could have resulted from cytokine-, oxidative stress- or endoplasmic reticulum (ER) stress- induced changes in gene expression and cell survival [Cnop *et al.*,

2005]. A rise in cytoplasmic free fatty acid levels leads to increased ceramide formation, which induces iNOS, resulting in NO mediated β-cell apoptosis [Cnop *et al.*, 2005; Shimabukuro *et al.*, 1997; Prentki and Nolan, 2006].

β-cell neogenesis (or regeneration) represents a major goal of therapy for diabetes. Successfully unraveling the origin of β-cells during pancreatic neogenesis could lead to restoration of β-cell mass in individuals with type 1 diabetes and enhanced β-cell compensation in type 2 patients. Therefore, it would be clinically relevant to investigate the transcription factors that are involved in the process of β-cell neogenesis and potential methods of manipulating these transcription factors.

4.1.2.3 β-cell neogenesis and transcription factors involved

Neogenesis is an important component of β-cell mass expansion during development. The mechanism of islet neogenesis remains poorly understood, despite its potential applications in regenerative or replacement therapies for the treatment of type 1 and type 2 diabetes.

β-cell neogenesis can occur through two pathways: (i) stem/progenitor cell activation and/or (ii) transdifferentiation of adult pancreatic cells [Xu *et al.*, 2008]. β-cell mass expansion derived from stem/progenitors cells, refers to the differentiation of a dormant cell expressing primordial markers of insulin producing β-cells. There is, however, still controversy around the existence and classification of such pancreatic stem cells [Abraham *et al.*, 2002; Dor *et al.*, 2004; Seaberg *et al.*, 2004]. Very little is known about these stem cells that reside within the islet itself [Zulewski *et al.*, 2001; Petropavlovskaia and Rosenberg, 2002] or in the pancreatic ducts [Bonner-Weir *et al.*, 2000 (a); Zhao *et al.*, 2007]. In contrast, transdifferentiation of adult cells refers to the ability of one mature cell phenotype to differentiate to another cell phenotype. The hypothesis here is that the new β-cells are derived directly from the transdifferentiation of mature, differentiated duct or acinar pancreatic cells or

through a process of dedifferentiation to a more primitive intermediate cell, followed by redifferentiation into an insulin-producing cell.

It is believed that all specialized endocrine as well as exocrine cells of the pancreas are derived from the same precursor cells which reside within the endoderm [Bouwens and Rooman, 2005; Percival and Slack, 1999; Wilson *et al.*, 2003]. Differentiation of endocrine progenitor cells into hormone-producing islet cells is tightly regulated by the chronological expression of numerous transcription factors. With *in situ* hybridization screening Zhou and colleagues (2007) identified more than 1100 transcription factors with cell-type-specific expressions in the embryonic pancreas. Of those, at least 20 transcription factors were found to be expressed in mature β-cells and their immediate precursors and 9 genes exhibited β-cell developmental phenotypes when mutated [Murtaugh and Melton, 2003; Jensen, 2004]. A combination of the transcription factors pancreatic and duodenal homeobox 1 (*Pdx1*), neurogenin 3 (*Ngn3*) and musculoaponeurotic fibrosarcoma oncogene homolog A (*MafA*) was found to be required in the differentiation of endocrine progenitors to hormone-producing β-cells [Zhou *et al.*, 2008].

The homeodomain transcription factor Pdx1 is one of the earliest genes expressed in the developing pancreas, which is essential for pancreas development and all pancreatic cells are thought to arise from a common Pdx1 expressing progenitor cell [Gu *et al.*, 2002]. In the differentiated β-cell, Pdx1 is a glucose responsive regulator of insulin gene expression [Petersen *et al.*, 1998]. When transduced Pdx1 in cultured pancreatic ducts interacted with the insulin gene promoter, the interaction triggered insulin gene expression [Noguchi *et al.*, 2003; Wilson *et al.*, 2003]. This suggests that ductal Pdx1 expression has effects on neighbouring cells within the pancreatic duct and then may induce the first steps of β-cell differentiation. Research done by Holland and colleagues (2005) demonstrated that when Pdx1 in adult mice was repressed, the expression of insulin and glucagon was impaired and this led to diabetes within 14 days. These findings confirm that Pdx1 is required for β-cell function in the adult pancreas [Holland *et al.*, 2005] and that Pdx-1 may have the capacity to reprogram extra-pancreatic tissue towards a

β-cell phenotype, which may provide a valuable approach for generating β-cells, suitable for replacing impaired β-cell function found in diabetes.

During pancreas development, all endocrine cell types are derived from progenitors that express the transcription factor Ngn3 [Gradwohl *et al.*, 2000]. Embryos lacking the Ngn3 transcription factor lack all endocrine cell types and die two to three days after birth due to diabetes [Gradwohl *et al.*, 2000]. According to research done by Xu and colleagues (2008), there is a 10-fold increase in dividing β-cell number, following partial duct ligation and that the appearance of these new β-cells correlates with activation of Ngn3. This suggests that Ngn3 plays a key role in β-cell regeneration.

MafA is a recently isolated β-cell-specific transcription factor that functions (in the presence of Pdx1) as a potent activator of insulin gene transcription in the developing β-cell [Kaneto *et al.*, 2007; Matsuoka *et al.*, 2004]. Under diabetic conditions, expression and activity of Pdx1 and MafA in β-cells are reduced, which inevitably leads to suppression of insulin biosynthesis and secretion. Together Pdx1 and MafA play an important role inducing β-cells and could be a therapeutic target for diabetes [Kaneto *et al.*, 2007].

4.1.2.4 Current therapies for type 2 diabetes

The mechanisms involved in the development of this disease are an active field of research that may bring about the development of newer and safer therapies. The current available therapies do not successfully enable patients with type 2 diabetes to reach glycaemic goals. Even with intensive treatment, patients with type 2 diabetes may face spikes in blood glucose levels after meals, weight gain and a loss of effectiveness of their treatments over time [Pearson, 2009; Moller, 2001]. The present therapies mainly rely on the approaches to reduce hyperglycaemia by means of oral hypoglycaemic agents.

Table 1: Current therapies used for managing hyperglycaemia in type 2 diabetes

Drug	Description of effects	Documented side-effects
Metformin	Suppresses gluconeogenesis and thus reduce hepatic glucose production [Correia *et al.*, 2008; Pearson, 2009]	Gastrointestinal side-effects, such as diaree and impaired digestion [Bosi, 2009]
Sulphonylureas	Stimulates insulin secretion by β-cells in a non-glucose dependent manner [Kar and Holt, 2008; Pearson, 2009]	Weight gain, hypoglycaemia, gastrointestinal distress, dermatological reactions, haematological changes, ocular problems inappropriate secretion of antidiuretic hormone [Salas and Caro, 2002]
Thiazolidinediones (TZD)	PPARγ agonist, which enhance insulin action primarily through indirect effects on lipid metabolism [Pearson, 2009; Edgerton *et al.*, 2009]	Osteoporosis [Meier *et al.*, 2009]

5. OBESITY AND CARDIOVASCULAR DISEASE

Numerous studies have linked obesity to an increased risk of developing cardiovascular disease. This is due to the multitude of risk factors associated with obesity, such as hypertension, diabetes and dyslipidaemia [Reaven 1988, Seidell *et al.*, 1996; Lopaschuk *et al.*, 2007]. Presently, cardiovascular diseases represent the most important health risk factors because they are responsible for more than 50% of total mortality [Ostadal, 2009]. According to projections made in 2009 by the WHO, cardiovascular diseases claim 17.1 million lives annually. The types of cardiovascular diseases include, ischaemic heart disease, cerebrovascular disease (or commonly known as a stroke), congestive heart failure, coronary artery disease and peripheral vascular disease.

Among these cardiovascular diseases, ischaemic heart disease (IHD) is the leading cause of death in the industrial world and according to the WHO will be the major global cause of morbidity and mortality by the year 2020 [Murray and Lopez, 1997].

5.1 Ischaemic heart disease

Ischaemic heart disease, or otherwise called myocardial ischaemia, develops as a consequence of a number of risk factors and always coexists with other disease states, which include systemic arterial hypertension and related left ventricular hypertrophy, hyperlipidemia, athrosclerosis, diabetes, insulin resistance, heart failure as well as ageing [Ferdinandy *et al.*, 2007]. IHD develops when there is an imbalance between the normal rate of oxygen delivery to the myocardium required for cardiac muscle contraction and the actual rate of oxygen delivery to the myocardium [Stanley, 2001]. Diabetic patients, both type 1 and type 2, have a 2-fold increased risk of sustaining myocardial ischaemia, as well as 2.4-fold increased mortality post-ischaemia [Haffner, 1999; Taegtmeyer *et al.*, 2002]. An increased susceptibility of the myocardium to ischaemia in the diabetic and dysmetabolic state leads to an

increased infarct size and this has been suggested to play a key role in the poor prognosis of myocardial infarction in type 2 diabetes mellitus patients [Song and Hardisty, 2008; Haffner, 1999].

Infarct size is an important determinant of mortality and heart failure after myocardial infarction [Burns *et al.*, 2002]. Pharmacological agents, such as insulin [Ryu *et al.*, 1999], urocortin [Brar *et al.,* 2002], atorvastatin [Bell and Yellon, 2003 (a)], bradykinin [Bell and Yellon, 2003 (b)], erythropoietin [Bullard *et al.*, 2005] and glucagon-like peptide 1 (GLP-1) [Bose *et al.,* 2005], which are known to activate PI3K/PKB/Akt signaling pathways, have all been shown to reduce necrotic tissue and thus protect against myocardial infarction.

In acute myocardial infarction, the primary event is most commonly a thrombus in an atherosclerotic coronary artery, which is due to a process termed atherosclerosis. Cytokines and FFA increase the production of fibrinogen and plasminogen activator inhibitor-1 (PAI-1) by the liver, which results in a pro-thrombotic state (refer to Figure 1.2). Fibrinogen is a clotting factor and the increased concentration thereof causes hypercoagulability, which is a potentially dangerous condition in which blood coagulates excessively. Studies have shown that hypertension, which has a strong association with obesity, has both a role in the genesis of atherosclerosis and in the extent and instability of the atherosclerotic plaque [Davies, 1991; Megnien *et al.*, 1996; Sipahi *et al.*, 2006].

Atherosclerosis is a progressive disease characterized by the accumulation of calcium crystals, smooth muscle cells, macrophages, fatty streaks and inflammatory mediators [Ross, 1999] in arteries, to form plaques. Plaques can increase in size to such an extent that they significantly reduce the vascular lumen (inside space of the artery) diameter, thus reducing the blood flow through an artery. However, the bulk of the damage occurs when these plaques become fragile and ruptures, leading to the expulsion of pro-thrombotic factors into the bloodstream. Ruptured plaques cause thrombi formation that can block blood flow or break off and travel to another part of the body, where it lodges into smaller arteries, capillaries or veins. If the thrombus occludes a blood vessel that feeds the heart, the myocardium below

the occlusion is left without sufficient oxygen supply to meet metabolic demand, leading to compromised myocardial viability and cardiac function [Ross, 1999; Opie, 1991].

Apart from large vessel disease and atherosclerosis, which are very common in diabetes, diabetic cardiomyopathy (DCM) is a distinct primary disease process, independent of coronary artery disease, which leads to heart failure in diabetic patients [Avogaro *et al.*, 2004].

5.2 Diabetic cardiomyopathy

Diabetes mellitus results in a marked increase in cardiac disease and this is partly due to diabetic cardiomyopathy. Diabetic cardiomyopathy can be defined as abnormal myocardial performance or structure in the absence of coronary heart disease and hypertension [Severson *et al.*, 2003; Shehadeh and Regan, 1995]. Rubler and colleagues (1972), who reported autopsy data from four patients with diabetic renal microangiopathy and dilated left ventricles in the absence of other common causes, made the first report of diabetic cardiomyopathy more than 30 years ago. The concept of diabetic cardiomyopathy remains slightly vague, despite nearly 40 years of basic and clinical research [Boudina and Abel, 2007]. This could be due to the lack of consensus over its definition and the myocardial abnormalities that are often overlooked.

The pathogenesis of this disease is indeed complex, however, alterations in cardiac energy metabolism have been proposed as contributing mechanisms [Jagasia and McNulty, 2003]. Hyperglycaemia is also considered to play a central part in the pathophysiology of this disease, because it can trigger several responses, both adaptive and maladaptive. Several epidemiologic studies [Palmieri *et al.*, 2001; Bella *et al.*, 2001; Liu *et al.*, 2001; Rutter *et al.*, 2003] suggest that there is an association between diabetic cardiomyopathy and the presence of cardiac hypertrophy and myocardial stiffness, both being independent of hypertension.

Finding treatments for diabetic patients with underlying heart diseases, presents as a challenge, due to the complexity of the pathophysiology and the poor prognosis of these comorbidities. Therefore, investigation of the cellular metabolism and mechanisms of interaction of these diseases is of particular interest.

5.3 Cardiac energy metabolism

The heart consumes more energy than any other organ, cycling approximately 6 kg of adenosine triphosphate (ATP) every day while circulating nearly 10 tons of blood throughout the body [Neubauer, 2007]. Under normal coronary blood flow, almost all (> 95%) ATP formation in the heart is generated during oxidative phosphorylation in the mitochondria and the remaining 5% is generated during glycolysis and guanosine triphosphate (GTP) formation in the citric acid cycle. The myocardium has a relatively low ATP content (± 5 μmol.g wet wt) and a high rate of ATP hydrolysis (± 0.5 μmol.g wet $wt^{-1}.s^{-1}$) at rest, which means that there is a complete turnover of the mitochondrial ATP pool approximately every 10 sec under normal conditions [Ingwall, 2002; Opie, 1991].

The cardiac muscle has a high demand for energy to maintain cellular homeostasis and normal contractile function. It is well known that under normal conditions the heart utilizes a host of different substrates, amongst which fatty acids are the major fuel for the generation of energy, with glucose following in second place. The heart switches between these substrates to accommodate different conditions, during both normal and pathological situations [Rodriques *et al.*, 1995; Stanley *et al.*, 1997 (b)]. Under circumstances of stress such as pathological hypertrophy or myocardial ischaemia, the substrate preference shifts from free fatty acids to glucose. This shift is capable of generating high-energy phosphates through both glycolytic flux as well as oxidation of the glycolytic intermediate, pyruvate [Bertrand *et al.*, 2008].

Diabetes has a marked influence on cardiac metabolism, due to the altered substrate supply, impaired insulin action and metabolic changes observed in the diabetic heart [Taegtmeyer *et al.,* 2002; Young *et al.*, 2002].

5.3.1 Fatty acid metabolism

In the healthy human heart fatty acids are the main suppliers of energy, at approximately 60 to 90% [Opie, 1998; Stanley *et al.*, 1997 (b); Balaban *et al.*, 1986]. The rate of fatty acid uptake by the heart is primarily determined by its plasma concentration [Lopaschuk *et al.*, 1994 (a); Wisneski *et al.*, 1987], which is in turn regulated by their net release from triglycerides in adipocytes. Free fatty acid concentration can vary in the range of 0.2 to 0.8 mM during the course of the day in healthy individuals. Under conditions of metabolic stress, such as ischaemia, diabetes, or starvation, concentrations can increase to levels up to about 1.0 mM [Lopaschuk *et al.*, 1994 (b)].

Insulin suppresses fatty acid levels, which is the reason why fatty acid levels are low after a meal. In contrast, during fasting, when insulin is low and catecholamines are high, the plasma fatty acid concentration is elevated, resulting in a high rate of fatty acid uptake and oxidation by the heart. Alternatively, noradrenaline increases fatty acid release from adipocytes during physical exercise, fasting or myocardial ischaemia. Diabetic patients (both types 1 and 2) have elevated levels of fatty acid, due to low insulin levels and/or resistance to the normal insulin-induced suppression of fatty acid release from adipocytes. This increase in fatty acid oxidation has an important suppressive effect on carbohydrate oxidation in the heart through inhibition of glycolysis in the cytosol and pyruvate dehydrogenase in the mitochondria, which in turn leads to impaired myocardial energy production and the accumulation of glycolytic intermediates and ceramide, which enhances apoptosis [Eckel and Reinauer, 1990; Liedtke *et al.,* 1988]. The toxic intermediates resulting from free fatty acid metabolism [Rodrigues *et al.*, 1998; Yazaki *et al.*, 1999] (so-called lipotoxicity) can impair myocyte calcium handling, worsening myocardial mechanics.

Fatty acids enter the cardiomyocyte by one of two processes: either by passive diffusion or by protein-mediated transport across the sarcolemma [Van der Vusse *et al.*, 2000], where it is converted to acyl-CoA. Fatty acids are oxidized via the process of β-oxidation in the mitochondria, where energy is released in the form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) for the electron transport chain and the subsequent formation of ATP by oxidative phosphorylation [Lopaschuk *et al.*, 1994 (a)].

The mitochondrial inner membrane is not permeable to long-chain acyl-CoA and the acyl-CoA must thus first be transferred from the cytosol into the matrix, by a carnitine-dependent transport system [Kerner and Hoppel, 2000]. The fatty acids must be converted to fatty acylcarnitine by the enzyme carnitine palmitoyl transferase 1 (CPT-1), in order to cross the inner mitochondrial membrane [Lopascuk *et al.*, 1994 (a)]. This process occurs between the inner and outer membrane of the mitochondria (refer to Figure 1.3A). Thereafter, carnitine acyltranslocase transfers this long-chain acylcarnitine across the inner mitochondrial membrane in exchange for free carnitine. Lastly, carnitine palmitoyltransferase II (CPT-II) regenerates long-chain acyl CoA in the mitochondrial matrix, where β-oxidation occurs.

β-oxidation involves four reactions and it is a process that repeatedly cleaves off two carbon acetyl-CoA units [Opie, 1998]. The first step is catalyzed by acyl-CoA dehydrogenase, followed by 2-enoyl-CoA hydratase and then 3-hydroxyacyl-CoA dehydrogenase. The final step is 3-ketoacyl-CoA thiolase, which regenerates acyl-CoA for another round of β-oxidation and releases CO_2 and acetyl-CoA for the citric acid cycle. Acyl-CoA dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase generate $FADH_2$ and NADH respectively and the acetyl-CoA formed from β-oxidation generates more NADH and $FADH_2$ in the citric acid cycle [Stanley *et al.*, 2005].

As mentioned above, the long-chain fatty acyl-CoA, can be converted to long-chain fatty acylcarnitine by carnitine palmitoyltransferase I (CPT-I) [Lopaschuk *et al.*, 1994 (a)], but it can also be esterified to triglyceride by glycerolphosphate acyltransferase [Coleman *et al.*, 2000; Lopaschuk *et al.*,

1994 (a)]. Under normal cardiac conditions, between 70 to 90% of the fatty acids that enter the cell are converted to acylcarnitine and immediately oxidized and the remaining 10 to 30% enter the triglyceride pool in the heart [Lopaschuk *et al.*, 1994 (a); Saddik and Lopaschuk, 1991].

These high plasma fatty acid levels could contribute to the impaired cardiac function observed in diabetes. Exposing the heart to high levels of fatty acids can cause accumulation of lipids (cardiac lipotoxicity) [Lee *et al.*, 2004; Wang and Unger, 2005]. This excessive accumulation of lipids within nonadipose tissue could lead to the increase in the intracellular pool of long-chain fatty acyl-CoA, thereby providing fatty acid substrate for nonoxidative processes, which can lead to cell dysfunction, insulin resistance and potentially apoptotic cell death [Lee *et al.*, 2004; Taegtmeyer *et al.*, 2002; Young *et al.*, 2002].

5.3.2 Glucose and lactate metabolism

Glucose and lactate supply approximately 10 to 40% of the cardiac energy requirements [Opie, 1998; Stanley *et al.*, 1997 (a); Gertz *et al.*, 1988; Wisneski *et al.*, 1985; Wisneski *et al.*, 1990]. The transport of glucose into the cardiomyocyte is via glucose transporters (mainly insulin-dependent-GLUT4 and to a lesser extent insulin-independent-GLUT1) and this is regulated by the transmembrane glucose gradient and the content of glucose transporters in the sarcolemma. Myocardial glucose use is substantially reduced in diabetes, due to diminished glucose uptake [Rodrigues *et al.*, 1998].

Glucose is utilized by the myocardium and processed in one of two ways: it is either stored as glycogen, or broken down through the process of glycolysis. The glycolytic pathway converts glucose-6-phosphate and NAD^+ to pyruvate and NADH, generating two ATP molecules for each glucose molecule (refer to Figure 1.3A). The first irreversible step of the glycolytic pathway is catalized by phosphofructokinase-1 (PFK-1), which is a key regulatory enzyme. PFK-1 makes use of ATP to generate fructose-1,6-bisphosphate and it is stimulated by adenosine diphosphate (ADP) and adenosine monophosphate (AMP).

PFK-1 can be inhibited by a fall in pH and the extent of the inhibition of PFK-1 depends on the ATP levels, with the inhibition being greatest when ATP levels are high. Citrate is a negative allosteric regulator of PFK-1 and links changes in mitochondrial oxidative metabolism to glycolysis. Philip Randle proposed that this accumulation of citrate is responsible for the decrease in glycolysis that occurs in various tissues when fatty acid oxidation increases [Garlind *et al.*, 1963; Newsholme and Randle, 1964; Randle *et al.*, 1968; Randle *et al.*, 1970].

The pyruvate formed during glycolysis can either be converted to lactate, decarboxylated to acetyl-CoA, or carboxylated to oxaloacetate or malate. Pyruvate decarboxylation is catalyzed by pyruvate dehydrogenase (PDH) [Randle, 1968]. PDH is inactivated by PDH kinase (PDK) and activated by PDH phosphatase [Randle, 1968; Randle and Priesman, 1996]. PDK has four isoforms, PDK-4 being the predominant form in the heart. The expression of PDK-4 is induced by starvation, diabetes and peroxisome proliferater activated receptor-α (PPARα) ligands [Harris *et al.*, 2001; Wu *et al.*, 2001] (refer to Figure 1.3B). During fasting as well as diabetes, there is high circulating lipid and intracellular accumulation of long-chain fatty acids and this enhances the PPARα-mediated PDK-4 expression, which leads to a greater phosphorylation inhibition of PDH and thus less oxidation of pyruvate derived from glycolysis and the oxidation of lactate [Horwich *et al.*, 2001; Wu *et al.*, 2001]. Glucose and pyruvate oxidation as well as the activity of PDH in the heart are reduced by elevated rates of fatty acid oxidation, such as occurs if plasma levels of free fatty acids are elevated. A well recognized fact is that the oxidation of glucose and lactate is strongly inhibited by high rates of fatty acid oxidation in the heart [Opie, 1998; Stanley *et al.*, 1997 (b); Wisneski *et al.* 1985; Wisneski *et al.* 1990; Randle *et al.*, 1963; Randle *et al.*, 1964].

An additional source of glucose-6-phosphate for the heart comes from intracellular glycogen stores. The glycogen pool in the heart is much smaller (± 30 μmol.g wet wt) compared to skeletal muscle (± 150 μmol.g wet wt) [Botker *et al.*, 1994; Opie, 1991] and it has a relatively rapid turnover rate [Henning *et al.*, 1996]. Glycogen concentrations can be increased by the

elevated supply of substrate and/or hyperinsulinaemia [Kruszynska *et al.*, 1991; Laughlin *et al.*, 1994; Stanley *et al.*, 1997 (a)]. Adrenergic stimulation (leading to increased cAMP and Ca^{2+}), a drop in ATP content and a rise in inorganic phosphate, which occur with ischaemia or intense exercise [Goldfarb *et al.*, 1986; Hue *et al.*, 1995; Stanley *et al.*, 1997 (a)] are all activators of glycogenolysis.

5.3.3 Ketone body metabolism

Under normal physiological conditions, the arterial plasma concentration of ketones (3-β-hydroxybutyrate and acetoacetate) is generally very low, which makes it a minor substrate for the myocardium. However, during starvation or poorly controlled diabetes the hepatic synthesis of 3-β-hydroxybutyrate and acetoacetate increases. This leads to elevated plasma ketone body concentrations and ketones becoming a major substrate for the heart [Avogaro *et al.*, 1990; Stanley *et al.*, 2005]. The myocardium of diabetic patients have a high rate of β-hydroxybutyrate uptake and fairly low rates of fatty acid uptake [Hall *et al.*, 1996], which suggests that in diabetic patients elevated plasma ketone concentrations act to inhibit fatty acid uptake and oxidation [Hasselbaink *et al.,* 2003].

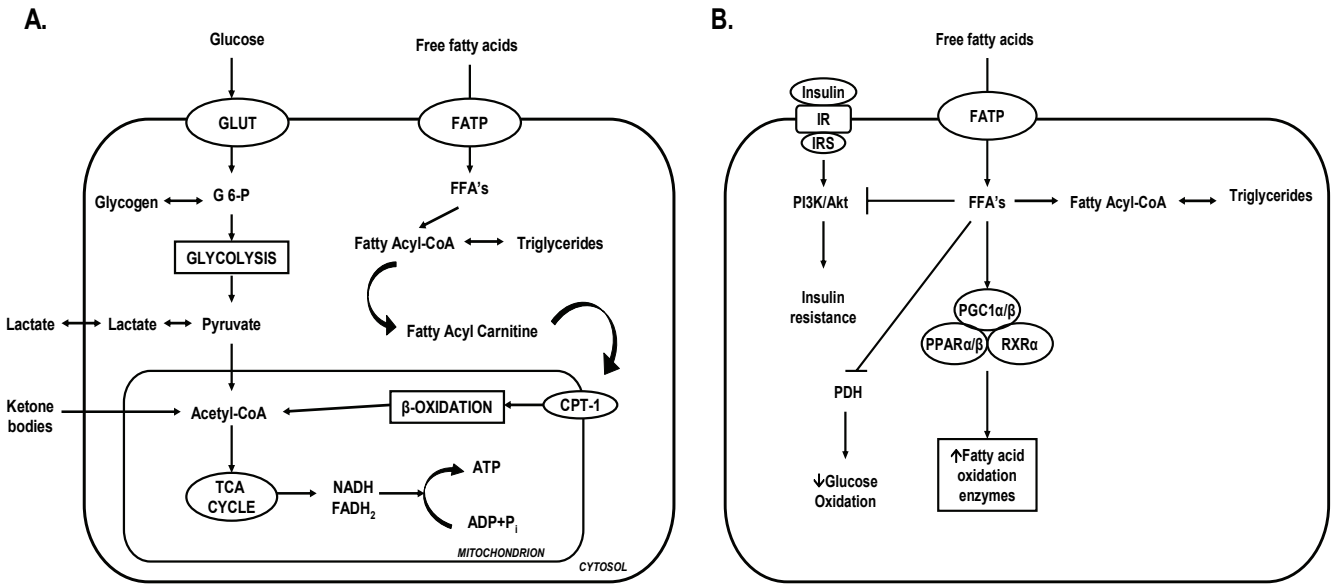


Figure 1.3: Schematic depiction of myocardial metabolism under conditions of (A) normal substrate concentration and (B) increased fatty acid availability. (A) Glucose is utilized by the myocardium in the form of G-6-P and processed in one of two ways: it is either stored as glycogen (which is also an additional source of G-6-P) or utilized in the process of glycolysis. The pyruvate formed during glycolysis can either be converted to lactate or decarboxylated to acetyl-CoA. Acetyl-CoA is used by the TCA cycle and NADH and $FADH_2$ produced, which leads to ATP formation (from $ADP+P_i$). FFA enters the cardiomyocyte where it is converted to acyl-CoA. Due to the mitochondia not being permeable to long-chain acyl-CoA, it is transferred to the matrix by a carnitine-dependent transport system. The acylcarnitine is converted by CPT-1 and enters the mitochondria where β-oxidation occurs. (B) Increased level of FFA availability results in (1) high rate of FFA uptake and oxidation, which inhibits the PI3K/PKB/Akt pathway, leading to the development of insulin resistance; (2) inhibition of PDH, resulting in decreased glucose oxidation and (3) overexpression of PGCα/β and PPARα/β, which results in fatty acid oxidation. Adapted from Chess and Stanley (2008). FATP: Fatty acid transporter; G-6-P: glucose-6-phosphate; CPT-1: carnitine palmitoyltransferase I; NADH: nicotinamide adenine dinucleotide; $FADH_2$: flavin adenine dinucleotide; ATP: adenosine triphosphate; ADP: adenosine diphosphate; IR: insulin receptor; PDH: pyruvate dehydrogenase; PGC-1: peroxisome proliferator-activated receptor gamma coactivator 1; PPAR: proliferator activated receptor

6. CELLULAR EFFECTS OF ISCHAEMIA/REPERFUSION LEADING TO CELLULAR INJURY

Damage incurred during myocardial ischaemia results from various factors that include the duration of ischaemia, oxygen demand by cardiomyocytes at the time of ischaemia and the degree of blood flow to the ischaemic area. Importantly, myocardial cell injury may not only be derived from the ischaemic incident itself, but also from detrimental effects that occur during the restoration of blood flow after the ischaemic incident. In the following sections the processes involved in ischaemia and reperfusion will be discussed. Figure 1.4 (page 39) is a schematic representation of the major cellular effects of ischaemia and reperfusion, which lead to irreversible forms of injury.

6.1 During ischaemia

During ischaemia, substantial changes occur in cardiac energy metabolism, as a consequence of reduced availability of molecular oxygen and metabolic substrates. A number of these metabolic changes are beneficial and may help the heart adapt to the ischaemic condition. However, most of these changes are maladaptive and contribute to the harshness of the ischaemic injury leading to cell death and contractile dysfunction [Rosano, 2008, Opie 1998].

The chain of events occurring during ischaemia is thought to be as follows: the lack of oxygen and metabolic substrates, lead to the dysfunction of mitochondrial metabolism, causing reduced aerobic formation (oxidative phosphorylation) of ATP [Hoffmeister *et al.*, 1986; Stanley *et al.*, 1997 (b); Liedtke, 1981]. This in turn triggers anaerobic glycolysis and disrupts normal cardiac cell function [Stanley, 1997 (a); Opie, 1998]. The first, short-term, defence of the heart is to achieve a new balance between the oxygen demand and supply by a combination of downregulation of contraction and aerobic energy production [Opie, 1998; Hochachka *et al.*, 1996]. Glycolysis can provide limited amounts of energy in the absence of oxygen.

The pyruvate, produced by glycolysis during ischaemia, is not so promptly oxidized in the mitochondria, leading to a high rate conversion of pyruvate to lactate in the cytosol and, in turn, a rise in tissue lactate content. Cellular homeostasis is dramatically disrupted when the ischaemic myocardium switches from the normal lactate uptake from the blood to the production thereof. Thus, anaerobic metabolism is linked with intracellular accumulation of inorganic phosphate, lactate and H^+, which leads to the fall in intracellular pH and a reduction in contractile work.

The SR Ca^{2+} uptake mechanisms are impaired due to the lack of ATP, which leads to the accumulation of intracellular Ca^{2+} [Gross *et al.*, 1999]. The fall in intracellular pH has numerous negative effects on the ability of cardiac muscle to maintain Ca^{2+} homeostasis and use the energy released from the breakdown of ATP to perform contractile work. This implies that, at low pH, for a given rate of ATP synthesis, more of the energy released from the breakdown of ATP contributes to the mechanism of regulating Ca^{2+} content in the cytosol and less to contractile work. Another negative consequence of ischaemia is the activation of the sodium-hydrogen exchanger (NHE) by intracellular acidosis, which leads to the accumulation of intracellular Na^+. This Na^+ overload is aggravated by inhibition of the sodium pump due to ATP depletion (refer to Figure 1.4A). These increased concentrations of solutes result in osmotic swelling that may cause sarcolemmal disruption. The extent of damage, such as the irreversible injury due to ischaemia, is time-dependent. Thus, the unrelieved lack of oxygen and metabolites will result in the pathological features of cell death.

6.2 During reperfusion

The view of the past decades was that the process of cell death occurred largely during ischaemia, as a result of the manifold consequences of ATP depletion. The hypothesis was that reperfusion is essential to restore ATP synthesis and so doing, to salvage those cells uninjured or reversibly injured by ischaemia. Currently, some researchers believe that all injury develops

during the ischaemic period, whereas others argue that reperfusion extends tissue injury. This phenomenon of “reperfusion injury” has been a subject of debate for the past three decades.

During reperfusion, the sudden reintroduction of oxygen and metabolites causes the reactivation of the mitochondrial electron transport chain. This process leads to massive free radical production, namely, reactive oxygen species (ROS) and reactive nitrogen species (RNS), in the presence of nitric oxide (NO). ROS and RNS cause oxidative and nitrosative damage to cellular structures, such as the SR, leading to Ca^{2+} release. Under conditions of restored ATP production, the activity of the Na^{+}/Ca^{2+} exchanger is restored, which leads to the extrusion of Na^{+} in exchange for Ca^{2+}. In addition, the SR Ca^{2+} release is also accentuated, which leads to increased cytosolic Ca^{2+} or cytosolic Ca^{2+} overload. The combined effects of Ca^{2+} accumulation in the mitochondrial matrix, ROS/RNS production and the increasing intracellular pH, due to H^{+} washout, favours the formation/opening of the mitochondrial permeability transition pore (mPTP), leading to further tissue damage [Opie, 1998] (refer to Figure 1.4A).

In the clinical treatment of myocardial infarction, early coronary reperfusion has proved to be the only way to limit infarct size [Ostadal, 2009]. There is thus no intervention, which is able to limit infarct development in the absence of reperfusion. However, the process of reperfusion is associated with further biochemical, structural and functional changes. This so-called “reperfusion injury” may lead to the death of cardiac myocytes that were still viable immediately before myocardial reperfusion. It is common to consider ischaemia/reperfusion injury as a composite entity with distinct components of injury, since it is true that reperfusion can never take place independently of ischeamia.

There is evidence from animal studies that reperfusion may contribute to irreversible cellular injury, leading to apoptosis and necrosis [Nilanjana and Tetsuya, 2000; Maulik *et al.*, 1998; Gottlieb *et al.*, 1994]. Oxygen free radicals, which are generated upon restoration of blood flow, are the main mediators of this reperfusion injury [Eefting *et al.*, 2004]. According to Piper and colleagues

(1998) there are three initial causes of immediate reperfusion injury, apart from oxygen radicals. They are re-energization, the rapid normalization of tissue pH and rapid normalization of tissue osmolality. Data obtained from patients with evolving acute myocardial infarction, such as in the Prague study, has demonstrated that reperfusion therapy may be beneficial, depending on the circumstances, such as how early reperfusion is applied [Widimsky *et al.*, 2003].

In recent years, studies have been devoted to the mPTP and its role in reperfusion injury. In the section below this role will be discussed.

6.3 The mitochondrial permeability transition pore (mPTP)

The mPTP is a voltage-dependent, high-conductance channel located in the inner mitochondrial membrane. It is widely accepted that mPTP opening contributes to the loss of viability, which is associated with post-ischaemic reperfusion. ROS accumulation, pH normalization and a rise in calcium ions, create an ideal scenario for mPTP opening [Di Lisa and Bernardi, 2006]. Since mPTP inhibition prevents reperfusion injury, this powerful protection, which is associated with postconditioning, could be attributed to a decreased probability of mPTP opening [Halestrap *et al.*, 2004].

According to the current knowledge of ischaemia/reperfusion injury and cardioprotection, the key determinant of cell death or survival is the opening or closing of the mPTP during reperfusion. For the past few decades it has been accepted that the mPTP is a proteinaceous complex comprising mainly of the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT) and cyclophilin D (CyP-D) [Halestrap and Brenner, 2003; Bernardi *et al.*, 2006]. However, recent evidence strongly opposes this notion. Based upon genetic deletion of ANT, VDAC and CyP-D [Baines *et al.*, 2005; Baines *et al.*, 2007; Kokoszka *et al.*, 2004; Krauskopf *et al.*, 2006] it appears that what were thought to be essential pore components are probably mPTP regulatory elements that are not required for pore formation [Juhasova *et al.*, 2008]. The

exact molecular composition of the pore is currently unknown and the subject of intense debate.

The mPTP acts as a voltage-dependent channel and is closed under normal physiological conditions when the inner membrane is impermeable to most solutes. However, under conditions of stress, the pore converts to an open state in the inner membrane that results in the loss of membrane impermeabililty and collapse of mitochondrial membrane potential and the subsequent release of cytochrome C. This occurence of mPTP opening during myocardial ischaemia/reperfusion and its contribution to ischaemia/reperfusion injury have been studied extensively during the last twenty years [Crompton *et al.*, 1987; Duchen *et al.*, 1993; Lemasters *et al.*, 1997; Halestrap *et al.*, 1998; Di Lisa *et al.*, 2001; Halestrap *et al.*, 2004; Di Lisa and Bernardi, 2006]. The exact mechanisms initiating apoptosis are still unknown. However, it seems likely that the opening of the mPTP during reperfusion (not during but after ischaemia) serves as a key mechanism of cell death to produce a pattern of reperfusion-induced necrosis [Crompton, 1999; Hajno´czky *et al.*, 2000; Bishopric *et al.*, 2001; Di Lisa *et al.*, 2001; Pacher and Hajno´czky, 2001; Halestrap *et al.*, 2004, Ferdinandy, 2007].

The current hypothesis is that specific manipulation during reperfusion of conditions that inhibit mPTP opening offers the potential to attenuate cell death through cardioprotective strategies. Thus, agents given at reperfusion may protect through a common mechanism of attenuating mPTP opening in early reperfusion. The concept that protective intervention can be used after the onset of ischaemia is thus receiving increasing attention and might be exploited in clinical settings [Piot *et al.*, 2008].

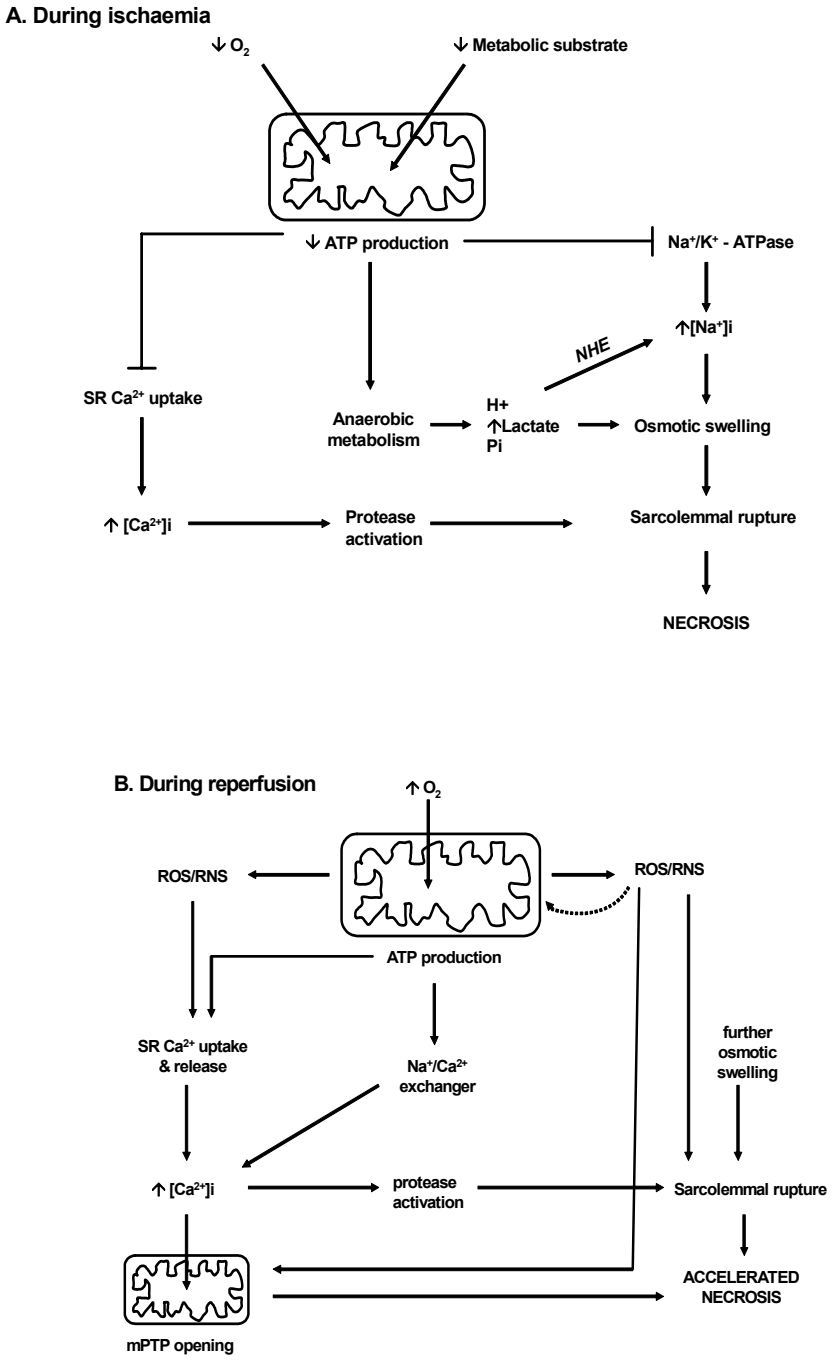


Figure 1.4: Major cellular effects of ischaemia and reperfusion leading to irreversible forms of injury. (A) During ischemia, reduced availability of molecular O_2 and metabolic substrates results in a deficit of high-energy phosphates. SR Ca^{2+} uptake mechanisms are impaired leading to intracellular Ca^{2+} accumulation. Anaerobic metabolism is associated with intracellular accumulation of inorganic phosphate, lactate and H^+. Activation of the NHE by intracellular acidosis leads to accumulation of intracellular Na^+. This Na^+ overload is exacerbated by inhibition of the sodium pump due to ATP depletion. Increasing intracellular concentrations of solutes results in osmotic swelling. The process of irreversible injury is time-

dependent and, in unrelieved ischemia, will result in the pathological features of necrosis. (B) The sudden reintroduction of molecular oxygen causes re-energization of mitochondria and reactivation of the electron transport chain with massive production of ROS, which may stimulate further ROS production (ROSinduced ROS release) and generation of RNS in the presence of NO. ROS/RNS cause oxidative and nitrosative damage to cellular structures, including the SR leading to Ca^{2+} release. Also, under conditions of restored ATP production, the activity of the Na^{+}/Ca^{2+} exchanger is restored, leading to the extrusion of Na^{+} in exchange for Ca^{2+}, and SR Ca^{2+} release is further accentuated by restoration of ATP leading to cytosolic Ca^{2+} overload. The combined effects of Ca^{2+} accumulation in the mitochondrial matrix, ROS/RNS, and increasing intracellular pH due to H^{+} washout favour the opening of the mPTP. The precise rate of injury or mode of cell death during reperfusion will be determined by the severity of changes during ischemia as well as by the extent of sarcolemmal fragility and disruption, which may be further exacerbated during reperfusion by further osmotic swelling and protease activity. Adapted from Fernandandy (2007).SR: sarcoplasmic reticulum; NHE: sodium-hydrogen exchanger; ROS: reactive oxygen species; RNS: reactive nitrogen species; mPTP: mitochondrial permeability transition pore

6.4 Cellular injury and infarction

At the onset of ischaemia, rapid structural changes occur in the myocardium. These may be either reversible or irreversible changes. These changes are considered reversible alterations if reperfusion of the tissue occurs rapidly. However, if this ischaemic incident lasts more than 20 to 30 min (in a rat heart), the result is irreversible tissue injury that is ultimately characterized by cell death [Reimer and Jennings, 1979; Jennings *et al.*, 1981]. Numerous factors that affect the onset and extent of irreversible injury in experimental models have been identified. Amongst these factors are the size of the area at risk of infarction, the duration of ischaemia [Reimer and Jennings, 1979; Ytrehus *et al.*, 1994], the degree of blood flow or residual flow through the infarct-related artery [Reimer and Jennings, 1979], myocardial temperature [Miki *et al.*, 1998] and heart rate may contribute to the rate of development of irreversible injury [Schulz *et al.*, 1995].

In the clinical setup, mortality related to myocardial infarction is closely related to the duration of the coronary occlusion. In the literature, it is well documented that certain cells subjected to ischaemia/reperfusion display signs of apoptosis. However, there is controversy as to the extent of apoptosis in ischaemia/reperfusion injury and the relationships between apoptosis and necrosis [Kajstura *et al.*, 1996; Misao *et al.*, 1996; Olivetti *et al.*, 1996; Saraste *et al.*, 1997; Baliga, 2001; Bishopric *et al.*, 2001].

6.4.1 Necrosis and apoptosis

In the human heart, cardiac failure is depicted by the progressive death of cardiomyocytes [Olivetti *et al.*, 1997]. According to the literature, there are two general mechanisms responsible for myocyte cell death during myocardial ischaemia/reperfusion, i.e. necrosis and apoptosis [Majno and Joris, 1995]. Up to about a decade ago, it was believed that all adult cardiomyocyte death was caused by necrosis, however, Narula and colleagues (1996) and Olivetti and colleagues (1997) provided some of the first evidence that apoptosis occurs in the myocardium of patients with end-stage dilated cardiomyopathy.

Cardiac cells undergoing necrosis show characteristically different morphological and biological features compared to those undergoing apoptosis. Necrosis is generally a rapidly occurring form of cell death that may trigger a significant inflammatory response. Changes include severe cellular and organelle swelling, denaturation and coagulation of cytoplasmic proteins and breakdown of cell organelles. In contrast, apoptosis is an energy-dependent process in which cell death follows a genetically controlled programmed sequence of events. Its main morphological features are: loss of cell membrane phospholipid asymmetry, condensation of chromatin and formation of cytoplasmic blebs. During the end stage of apoptosis, cellular fragments form into membrane-bound apoptotic bodies that confine intracellular content and prevent initiation of an inflammatory response [Haunstetter and Izumo, 1998, 2000; Bartling *et al.*, 1998; MacLellan and Schneider, 1997].

It might be possible that both apoptosis and necrosis are activated in the myocardium and may thus co-determine the final degree of lethal myocardial injury after ischaemia and reperfusion [Piper *et al.*, 1998; James, 1998].

6.4.2 Cellular survival: The role of PI3K/PKB/Akt pathway

PI3K/PKB/Akt signaling cascade is seen as the main prosurvival pathway [Duronio, 2008; Baines *et al.*, 1999]. The PI3K/PKB/Akt pathway appears to both negatively regulate factors that promote the expression of death genes and positively regulate factors that induce survival [Duronio, 2008; Parcellier *et al.*, 2008; Barthwal *et al.*, 2003; Brunet *et al.*, 1999].

Apoptosis, which was discussed in section 6.4 (page 40), refers to a complex set of events occurring within a cell that eventually leads to cellular destruction initiated by proteolytic enzymes known as caspases [Earnshaw *et al.*, 1999; Budihardjo *et al.*, 1999]. The ability of all cells to undergo apoptosis is suppressed by survival signals induced by factors within their immediate environment. PKB/Akt is crucial in cell survival. This can be seen in studies done where dominant negative alleles of PKB/Akt reduced the ability of growth factors and other stimuli to maintain cell survival, whereas activated PKB/Akt or over-expression of the wild-type, could rescue cells from apoptosis induced by various stress signals [Kennedy *et al.*, 1997; Kulik *et al.*, 1997].

Activated PKB/Akt has the ability to phosphorylate two classes of substrates that are known to be implicated in the life/death cycle of cells: (1) the anti-apoptotic substrates which, when phosphorylated, are activated and contribute to survival and (2) the pro-apoptotic substrates which, when phosphorylated [Franke *et al.*, 2003], become inactive and cell death can thus not occur. The PI3K/PKB/Akt pathway has been shown to play a vital role in myocardial protection against ischaemia/reperfusion injury in all species studied, both *in vivo* and *in vitro* [Hausenloy and Yellon, 2004; Mocanu and Yellon; 2007].

6.4.3 Autophagic cell death

During the past few years, another form of cell death, autophagic cell death, has drawn considerable attention [Edinger and Thompson, 2004]. Autophagy is a catabolic process involving the degradation of the cell's own components through lysosomal mechanisms. Autophagy has been recognised in a number of cardiac disorders, such as ischaemia and cardiac hypertrophy. This mechanism seems to help maintain a balance between the synthesis, degradation and subsequent recycling of cellular products [Goswanni and Das, 2006]. Thus, stimulation of autophagy may have cardioprotective effects. However, unlike in apoptosis, in which families of cysteine proteases and a number of other regulatory proteins have been identified, the mechanism of autophagic cell death remains unclear.

CHAPTER 2
Materials and Methods

2.1 Animal care

Throughout this study age and weight matched male, Wistar rats were used. All animals were housed in a temperature-controlled room (22 °C – 24 °C) and kept on a 12-hour light/dark cycle. Animals had free access to standard laboratory rat chow and water until the time of experimentation. The animals were housed at the University of Stellenbosch Central Research Facility, receiving humane care in accordance with the principles of the "Guide for care and the use of laboratory animals" published by the US National Institutes of Health (NIH publication no 80 - 23, revised 1985). The project was approved by the Ethics committee of the Faculty of Health Sciences, University of Stellenbosch. Ethics approval numbers are P05/11/013 for antidiabetic research and P07/11/020 for cardiovascular research.

2.2 Animal models

2.2.1 Streptozotocin-induced type 1 diabetes rat model

As soon as the rats reached the desired weight (230 – 250 g), they were randomly divided into two groups: control (n = 16) and diabetic (n = 24). Type 1 diabetes was induced with a single, intra-peritoneal injection of freshly prepared streptozotocin (STZ) (Sigma Aldrich, St Louis, MO) in citrate buffer (pH 4.5) and injected at 40 mg/kg body weight. The control animals were injected with buffer. This model has previously been shown to result in a graded diabetic response, where the animals retain a portion of their pancreatic function and do not suffer complete β-cell ablation [Arison and Fendale, 1967; Junod *et al.*, 1969; Brondum *et al.*, 2005].

One week post-STZ injection the animals were fasted overnight, blood collected via a once-off tail prick and plasma glucose concentration measured using a glucometer (GlucoPlus™, Montreal, Canada). Animals with fasting blood glucose levels of > 10 mmol/L were considered type 1 diabetic. From the 24 original animals, 9 animals had blood glucose levels exceeding 10 mmol/L. The 15 animals with lower blood glucose levels were excluded from the study. For both the control as well as the diabetic group, half of the animals (i.e control: n = 8 and diabetic: n = 5) were placed on Diavite™ treatment (25 mg/kg/day). This treatment continued for 8 weeks (refer to Figure 2.1 for a schematic representation of division into groups and Figure 2.2 for a diagram outlining the time-line of STZ experiment).

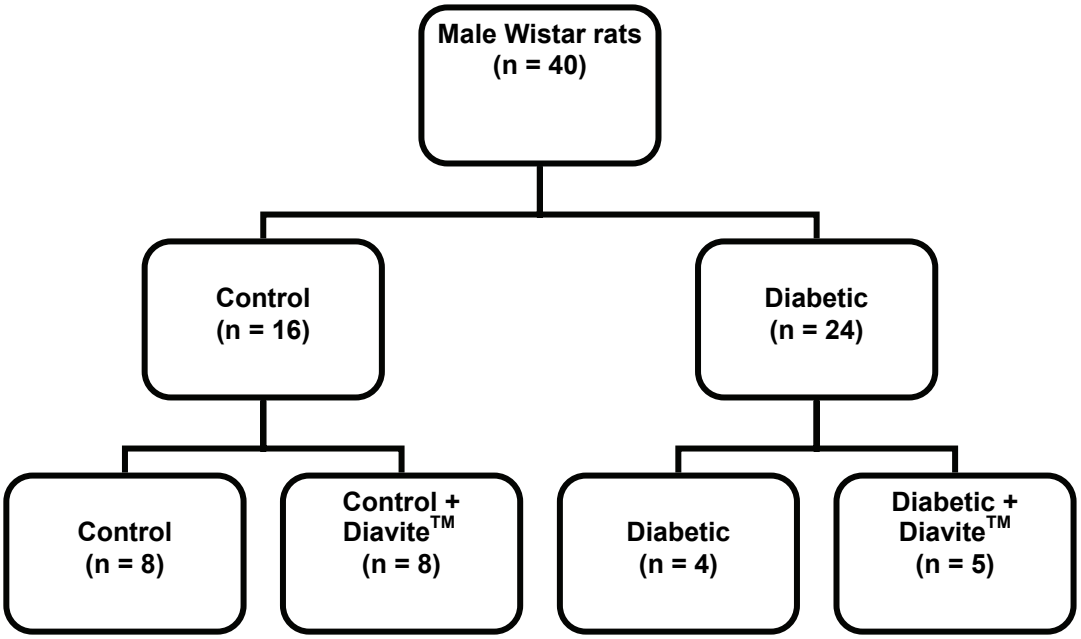


Figure 2.1: Schematic representation of the division into groups of STZ-induced type 1 diabetes animals

Diavite™ was weighed daily for each animal in the treatment group and set in a mixture of commercially available gelatine/ jelly cubes of 1 ml volume. This was fed to each animal, individually, to ensure absolute compliance and dose

control. Dosages of Diavite™ were calculated based on the normal daily dosage prescribed by the producers, which is 7 g per adult. To determine the dose for rats, we made our calculations assuming the average daily weight of an individual as 70 kg.

After an overnight fasting period (8 weeks after the onset of treatment), the animals were weighed and then anaesthetized with an intra-peritoneal injection of sodium pentobarbital (80 mg/kg). The animals were continually monitored until total loss of consciousness was reached, as indicated by a total lack of response after a foot pinch. The hearts were then removed, snap-frozen and stored in liquid nitrogen for further analysis. Blood samples were collected from the abdominal cavity and subsequently subjected to centrifugation (microcentrifuge at 1000 g at 4°C for 10 min), whereafter the plasma aliquots were stored at −80 °C until assays were done. Serum insulin levels were determined via a Coat-A-Count® Radioimmunoassay (RIA) kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA). The pancreata were also removed, stored in 4% phosphate buffered formalderhyde and sent for pancreatic analysis at the Medical Research Council (MRC) in Cape Town, South Africa. Histological data to be discussed in section 2.7 on page 59.

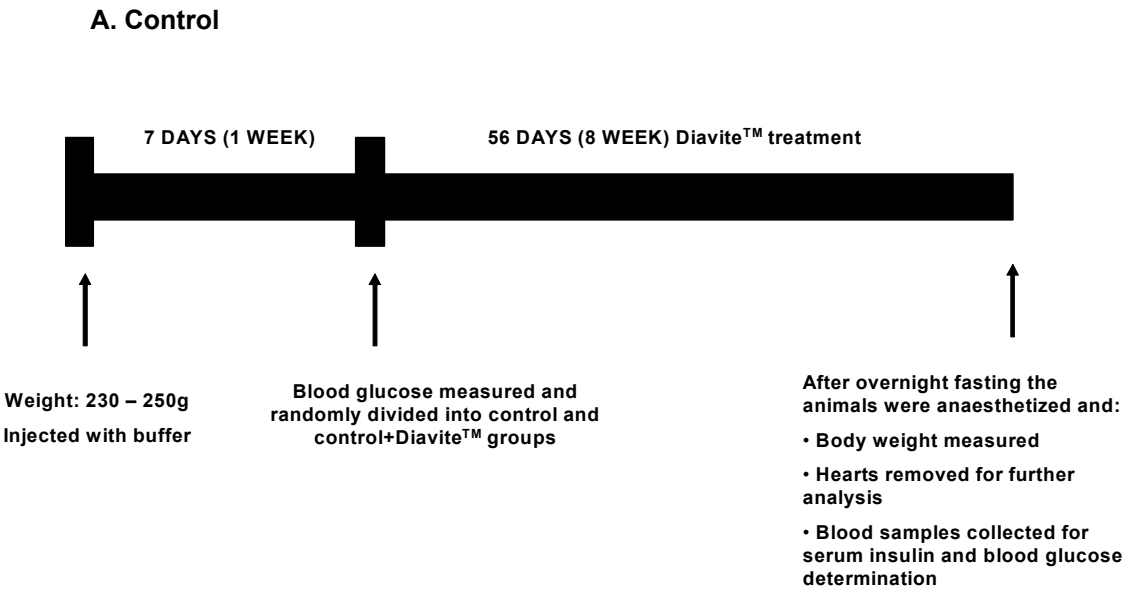


Figure 2.2: Diagram outlining the time-line of STZ experiment. (A) control and (B) diabetic group

2.2.2 Diet-induced obese insulin resistant rat model

2.2.2.1 Special diets

We utilized a model of diet-induced-obesity (DIO) [Pickavance *et al.*, 1999] with the concurrent development of insulin resistance. This model is one of hyperphagia-induced obesity and it has been characterized in our laboratory and shown to be physiologically relevant and comparable to the human equivalent of insulin resistance as a result of obesity [Du Toit *et al.*, 2005]. In the DIO model, the diet of the adult rats are changed similarly to the changes experienced by humans, changing from the more frugal, rural diet to a Westernized, fast food diet. This high caloric diet consists of normal rat chow supplemented with sucrose and condensed milk, resulting in an elevated fat and carbohydrate intake coupled to a low protein intake (refer to Table 2.1 for the nutritional composition of the control versus DIO diet).

The control group of animals received normal unsupplemented rat chow.

Table 2.1 Macronutrient composition (% total energy value) of diet consumed by control versus diet-induced obese (DIO) animals

	Control	DIO
Carbohydrates	**60%**	**65%**
Fat	**10%**	**16%**
Protein	**30%**	**19%**
	371 ± 18 kJ/day	570 ± 23 kJ/day

2.2.2.2 Division into groups

Male Wistar rats weighing in at 180 - 200 g were randomly divided into two groups: control (n = 45) and DIO (n = 45) and placed on their corresponding diets for a period of 8, 12 and 16 weeks respectively (refer to Figure 2.2 for schematic representation of division into groups as well as sample size). The

control group received the standard rat chow and the DIO group received the high caloric diet. The 8-week animals were not placed on the Diavite™ treatment. However, the 12 and 16-week animals was on their different diets (control and DIO respectively) for 8 weeks, where after half of each group (i.e. n = 9) was placed on the Diavite™ (25 mg/kg/day) treatment for the remaining 4 and 8 weeks, respectively.

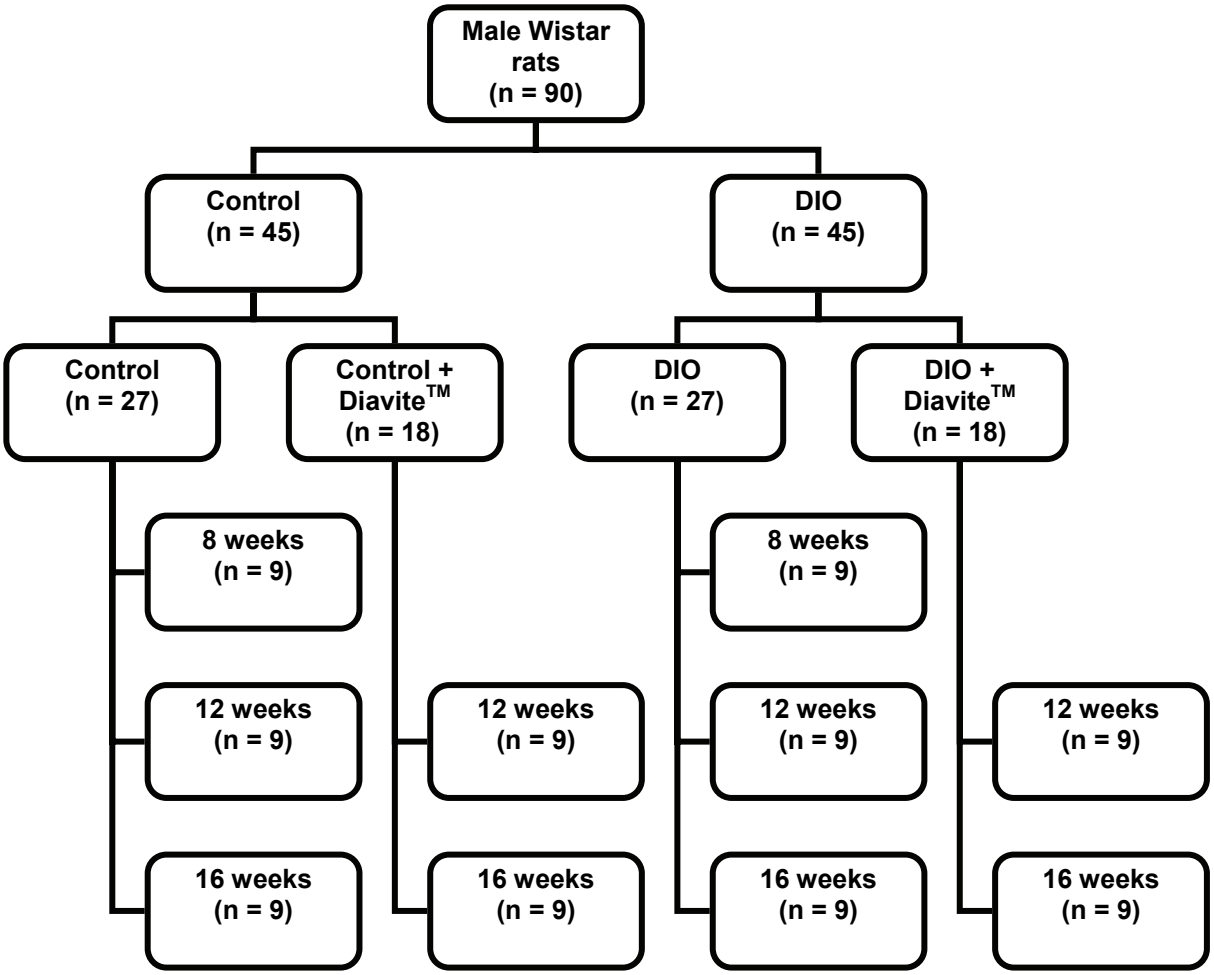


Figure 2.3: Schematic representation of the division into groups of DIO insulin resistant animals. DIO: diet-induced obesity

2.3 Isolated rat heart perfusion technique

2.3.1 Retrograde perfusion (Balloon model)

Following the 8, 12 and 16 week period on the feeding programme the rats were anaesthetized by intra-peritoneal injection of sodium pentobarbital at a dose of 80 mg/kg. Rats were continually monitored until total loss of consciousness was reached, as indicated by a total lack of response after a foot pinch. Body weights were recorded was soon as the animals lost consciousness. At the point of sacrifice, non-fasting blood was collected for blood glucose and serum insulin measurements, which was determined by means of a glucometer (GlucoPlus™) and a Coat-A-Count® Radioimmunoassay (RIA) kit (method discussed in section 2.8.2 below), respectively. In addition, intra-peritoneal fat was removed and the weight thereof recorded.

The hearts were excised and placed in ice-cold Krebs-Henseleit buffer before being mounted on a Langendorff perfusion apparatus via the aortic cannula. Retrograde perfusion was resumed within 1 to 2 minutes after excision of the heart, during which all excess adipose and connective tissue were trimmed off. Nutrient-rich, Krebs-Henseleit buffer was used as perfusate and it contained: 119 mM NaCl; 24.9 mM $NaHCO_3$; 4.74 mM KCl; 1.2 mM KH_2PO_4; 0.6 mM $MgSO_4.7H_2O$; 0.6 mM Na_2SO_4; 1.25 mM $CaCl_2.2H_2O$ and 10 mM glucose. All these chemicals were purchased at Merck (Pty) Ltd - South Africa. The buffer (pH = 7.4) was oxygenated by gassing it with 95% O_2/ 5% CO_2. Pressure (100 cmH_2O) and temperature (37 ± 1 °C) were maintained throughout the experiment and a temperature probe, which was inserted into the coronary sinus, continuously monitored the change in temperature. The pressure of the solution caused the aortic valve to shut and the perfusate to be forced into the coronary vessels. This allowed the heart to beat for several hours. In this model, a water-filled latex balloon coupled to a pressure transducer (Viggo Spectromed) was inserted into the left ventricle of the heart, to measure changes in developed pressure. This technique allowed the examination of cardiac contractile strength and performance, heart rate and vascular effects without the neuronal and hormonal complications of an intact

animal model. Thus, the functional performance, before and after the ischaemic insult, was computed in terms of the heart rate (HR), left ventricular developed pressure (LVDP: left ventricular systolic pressure – left ventricular diastolic pressure) as well as the rate pressure product (RPP). The RPP is calculated as the cumulative effect of the change in left ventricular developed pressure and the heart rate (LVDP x HR). The changes in these parameters were continually recorded on a computerized system for the entire duration of the experiment.

The perfusion protocol of the isolated rat hearts consisted of a 30 min stabilization period, which was followed by a 35 min regional ischaemic incident and completed with a 120 min reperfusion period. All phases taking place at a constant temperature and pressure.

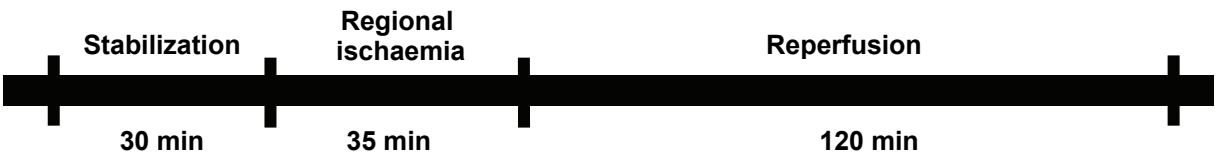


Figure 2.4: General perfusion protocol

2.3.2 Application of regional ischaemia

Regional ischaemia is used when infarct size needs to be determined. A silk suture was inserted around the left coronary artery, in such a way that it could be loosened to allow for reperfusion when needed. Ischaemia, which represents a heart attack, was induced by occluding the left anterior descending artery (for a period of 35 min) by tightening the suture. The successful occlusion was confirmed by a reduction in coronary flow. Following the 35 min period of regional ischaemia, the snare on the coronary artery was released and reperfusion was allowed for 120 min.

2.3.3 Infarct size determination

At the end of the experimental protocol, the silk suture, around the coronary artery, was tightened securely and 5 ml 0.5% Evans Blue suspension slowly infused into the aorta. This was done to delineate the area at risk of infarction. The hearts were removed from the system and frozen overnight before being sectioned into ± 2 mm thick slices. After defrosting, the slices were stained by incubation at room temperature for 15 min in 1% w/v triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4), which is a well-recognized dye technique. TTC staining results in the viable tissue staining a “brick-red” colour, as the tetrazolium salts react with the dehydrogenases in the cells and the infarcted tissue stains a pale-white since they lack the enzymes with which the TTC reacts [Han and colleagues, 2008]. Slices were then fixed in 10% v/v formaldehyde solution for 2 hours to stop all chemical reactions and enhance the contrast between stained viable tissue and unstained necrotic tissue. The blue area represents the viable and undamaged tissue, the white area presents the unstained, necrotic tissue and the “brick-red” area represents the area at risk. Each slice was mounted between two plates of transparent perspex of a known distance apart and traced onto a transparent film. The images were imported into the UTHSCSA ImageTool graphics package (University of Texas Health Science Center at San Antonio, Texas) and the area at risk of infarction (AR) as well as the actual size of infarcted tissue (IFS) determined using planimetry. The UTHSCSA ImageTool graphics package is available from the internet at http://ddsdx.uthscsa.edu/dig/itdesc.html. The infarct size was expressed as the percentage of the risk zone infarcted.

2.4 Western blot analysis

2.4.1 Sample preparation

Separate groups of animals were used for the determination of infarct size and protein expression. Animals were placed on the same control and DIO diet (with and without Diavite^{TM}), as described in section 2.2.2.1 of Materials and Methods for a period of 16 weeks. At the end of the feeding programme the animals were sedated as described in section 2.3.1 of Materials and Methods, their hearts isolated, washed in ice-cold Krebs-Henseleit buffer and the ventricles snap-frozen using Wollenberger tongs, pre-cooled in liquid nitrogen. After the hearts had been snap-frozen they were submerged into and stored in liquid nitrogen until later use. Protein expression determination and quantification analysis were done as follows:

2.4.2 Protein extraction

The proteins of interest were extracted from the cardiac tissue by means of a lysis buffer that contained: 2 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1 mM β-glycerophosphate, 2.5 mM tertasodiumpyrophosphate, 1 mM sodium orthovanadate (Na_3VO_4), 1% Triton X-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 50 µg/ml phenylmethyl sulfonyl fluoride (PMSF). Frozen heart tissue (± 200 mg) (section 2.4.1) was pulverized and homogenized, using a Polytron PT-10 homogenizer (2 x 4 sec, setting 4) in 0.7 ml cold lysis buffer. The homogenate was left to stand on ice for 15 minutes to allow digestive processes to take place. After 15 minutes, the homogenate was transferred to Eppendorf tubes and samples were subjected to centrifugation at 1000 g for 10 min at 4 °C, where after the supernatant was collected in a separate set of Eppendorf tubes.

The protein content of each sample was measured by means of the Bradford (1976) protein method. Bradford solution contained: 0.6 mM Coomassie Brilliant Blue G-250, 95% ethanol and 85% (w/v) phosphoric acid. Colour

development (absorbance) was read at 595 nm against a blank and sample values were determined from a standard curve generated from bovine serum albumin (BSA) of known concentrations. This sensitive method is suitable for measuring microgram quantities of proteins. The supernatant was then diluted in Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptothanol, 0.0004% bromphenol blue and 0.125 M Tris-HCl) to contain equal amounts of protein per volume unit [Laemmli, 1970]. The samples was boiled for 5 min and aliquots stored at -80 °C.

2.4.3 Protein separation

All stored aliquots were boiled for 5 min and samples were subjected to centrifugation at 15000 rpm for 2 min. Of each sample, 60 µg was loaded in a stacking polyacrylamide gel and separated according to their molecular weights by subjection to a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in running buffer (refer to Table 2.2 for the tabular representation of the Western blot analysis). The running buffer contained: 50 mM Tris, 384 mM glycine and 1% SDS. A standard Bio-RAD Mini-Protean III system was used. A protein ladder, obtained from Fermentas Life Sciences, was utilized as marker to identify the molecular weights of the proteins of interest.

The proteins separated within the SDS-gel were then transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon^{TM} P, Millipore) with an applied electrical current of 200 V for 1 hour, in a tank filled with transfer buffer. The transfer buffer consisted of 25 mM Tris, 192 mM glycine and 20% methanol. At the end of the transfer period, the membranes were immersed in fresh methanol and left to air dry. This was done so that the membranes could be stained with 5% Ponçeau Red in acetic acid (reversible protein stain), for visualization of proteins and to confirm whether adequate transfer did in fact occur.

Once the Ponçeau Red was rinsed off, the non-specific binding sites on the membranes were blocked by gently incubating them in fat-free milk, made up in a TBS-Tween solution (Tris-buffered saline (TBS) plus 0.1% Tween 20), for between 1 and 2 hours, at room temperature on a shaker. At the end of the "blocking" period, the membranes were thoroughly washed in the TBS-Tween solution. These membranes were then probed with primary antibodies directed against: GLUT1, GLUT4, total and phospho-PKB/Akt (Ser^{473}), total and phospho-PTEN (Ser^{380}/Thr^{382}/Tyr^{383}), total and phospho-PI3K p85 (Tyr^{458})/p55 (Tyr^{199}) and total IRβ and left to incubated overnight at 4 °C. The antibodies were diluted as stated in Table 2.2.

2.4.4 Immunodetection of protein

After the overnight primary antibody incubation, the membranes were thoroughly washed in TBS/Tween and incubated in an anti-rabbit immunoglobulin G, Horseradish-peroxidase conjugated secondary antibody (from donkey), purchased at Amersham life Science (Sandton, Johannesburg), for 1 hour at room temperature on a shaker. The secondary antibody was diluted as stated in Table 2.2. This conjugated antibody now bound to the already bound primary antibody. To remove the excess secondary antibody, the membranes were washed extensively in TBS/Tween and kept moist.

Proteins were visualized by covering the membrane with enhanced chemiluminescence (ECL) detection reagent (from Amersham life Science, Sandton, Johannesburg) for 1 minute and then exposing it to an autoradiography film (Hyperfilm ECL, RPN 2103). The Horseradish-peroxidase reacts with the detection reagent in a luminescence reaction and light emission that results, is captured on the radiography film. Band intensities were then densitometrically quantified using UN-SCAN-IT^{TM} (version 5.1, Silkscience) image analysis software.

For comparison purposes, samples from negative control hearts were always included in each blot and used for normalization of the unknown samples (i.e calculation of the ratio between the sample and negative control). Normalized data was expressed in arbitrary units (AU).

In all instances the membranes were stripped, by incubating for 5 min in 0.2 M NaOH and reblotted with antibody against β-tubulin (1:1000, Cell Signaling Technology, Beverly, MA) to verify the uniformity of protein load and the transfer efficiency across the test samples.

Table 2.2: Tabular representation of Western blot analysis

Protein	**Molecular weight**	**Polyacrylamide stacking gel**	**Seperation gel**	**Blocking solution dilutions**	**Primary antibody dilutions**	**Secondary antibody dilutions**
GLUT1	± 55 kd	10%	4%	2.5% milk in TBS/Tween	20µl/5ml in 1% milk	3µl/20ml in 2.5% milk
GLUT4	± 45 kd	10%	4%	5% milk in TBS/Tween	5µl/5ml in TBS/Tween	5µl/20ml in 2.5% milk
PKB	± 63 kd	10%	4%	5% milk in TBS/Tween	5µl/5ml in TBS/Tween	5µl/20ml in 2.5% milk
PTEN	± 54 kd	10%	4%	5% milk in TBS/Tween	5µl/5ml in TBS/Tween	5µl/20ml in 2.5% milk
p85	± 85 kd	10%	4%	5% milk in TBS/Tween	5µl/5ml in TBS/Tween	5µl/20ml in 2.5% milk
IRβ	± 98 kd	7.5%	4%	5% milk in TBS/Tween	5µl/5ml in TBS/Tween	5µl/20ml in 2.5% milk

2.5 Preparation of ventricular cardiac myocytes

Adult ventricular cardiomyocytes were prepared from a separate group of 16 week treated animals. Cardiomyocytes were isolated from animals on the DIO as well as the control diet, with or without Diavite^{TM} (as described in section

2.2.2.2) using isolation methods previously described by Fischer and collegues (1991) and subsequently modified in our laboratory [Huisamen *et al.*, 2001]. Hearts were excised from fully anaesthetized animals, as described in section 2.3.1 and placed in ice-cold Krebs-Henseleit buffer. After isolation, hearts were cannulated via the aorta and retrogradely perfused, at a temperature of 37 ^{o}C, with a calcium free HEPES buffer ("Solution A" containing: 6 mM KCl, 1 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 1.4 mM $MgSO_4$, 128 mM NaCl, 10 mM HEPES, 5.5 mM D-glucose and 2 mM pyruvate, pH 7.4) for five minutes to rinse out the blood. The perfusate was continually gassed with 100% O_2. The next step was perfusion, in a re-circulating fashion, with a HEPES digestion buffer ("Solution B" containing: "Solution A" + 0.7% fatty acid free BSA + 1 mg/ml collagenase + 18 mM 2,3 butanedione monoxime (BDM)) for 15 min. 50 µl of $CaCl_2$ (100 mM) was re-administered at 20 and again at 25 minutes of total perfusion time, respectively. Successful digestion was confirmed after perfusate no longer dripped from the heart, but flowed continously. After digestion, hearts were removed from the perfusion apparatus and the ventricles carefully removed from atria.

The ventricular tissue was then gently torn apart and incubated in a post-digestion buffer (50 ml "Solution C" containing: 50% of "Solution A" + 50% of "Solution B" + 1% BSA + 1% fatty acid free BSA + 0.2 mM $CaCl_2$) for 15 min at 37 ^{o}C in a shaking waterbath (180 strokes/min). A step-wise re-administration of calcium for a period of 5 minutes followed until the final concentration of 1.25 mM was reached (4 x 100 µl and 1 x 125 µl of 100 mM $CaCl_2$). Thereafter, the tissue was filtered through a nylon mesh (200 x 200 µm) and gently samples were subjected to centrifugation at 100 rpm for 3 min. The cell pellet was resuspended in Solution D ("Solution D" containing: "Solution A" + 2% fatty acid free BSA + 1.25 mM $CaCl_2$) and the cells allowed to settle for 5 min under gravity through the 2% BSA solution into a loose cell pellet. Only live, healthy cardiomyocytes will settle. The supernatant, containing dead or dying cells, was aspirated and the cell cell pellet obtained was resuspended in Solution D and left to stabilize on a slow rotator at room temperature for two hours. After the 2 hours, the cells were allowed to settle under gravity and the supernatant gently removed. The cells were then

washed with Solution E ("Solution E" containing: "Solution A" (minus D-glucose and pyruvate) + 2.0% fatty acid free BSA + 1.25 mM $CaCl_2$) and allowed to settle under gravity, whereafter the supernatant was removed and the cell pellet used for 2-deoxy-D-^{3}[H] glucose (2DG) uptake experiments as discussed below in section 2.6.

The HEPES, pyruvate and BDM were all obtained from Sigma-Aldrich (St Louis, MO), the D-glucose was obtained from Merck (Pty) Ltd - South Africa, the Collagenase Type II was purchased from Worthington Biochemical Corporation (Lakewood, NJ) and the BSA was obtained from Roche (Cape Town).

2.6 Determination of 2-Deoxy-D-^{3}[H] glucose (2DG) uptake by cardiomyocytes

The cell's ability to accumulate 2DG was measured as described previously [Fischer *et al.*, 1991; Donthi *et al.*, 2000; Huisamen *et al.*, 2001].

The cardiomyocyte cell pellet (section 2.5) was suspended in an oxygenated medium that contained: 6 mM KCl, 1 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 1.4 mM $MgSO_4$, 128 mM NaCl, 10 mM HEPES, 1.25 mM $CaCl_2$ and 2% fatty acid free BSA (pH 7.4). Aliquots (approximately 0.5 mg protein) were assayed in a total volume of 750 µl and were left to equilibrate for 15 min in a shaking waterbath (180 strokes/minute) at 37 °C, with or without phloretin (400 µM). An assay in the presence of phloretin was included to measure non-carrier mediated glucose uptake. Cells were then stimulated with or without 1, 10 or 100 nM insulin for 30 min in duplicate, whereafter they were incubated with 1.5 µCi/ml 2DG (PerkinElmer, Boston) in a final concentration of 1.8 µM deoxyglucose for 30 min, to allow for glucose uptake. The reaction was stopped by the addition of 50 µl (final concentration of 400 µM) phloretin in order to stop carrier-mediated (GLUT1 and GLUT4) glucose uptake. Following a 1 min microfuge at 1000 g, this 2DG containing supernatant was aspirated and cells were washed twice with a basic HEPES buffer that contained: 6 mM

KCl, 1 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 1.4 mM $MgSO_4$, 128 mM NaCl and 10 mM HEPES. The cell pellet was then dissolved in 0.5 ml of 1 N NaOH at 70 °C in a water bath for 30 to 40 min; whereafter 0.5 ml of dH_2O was added.

A 50 µl aliquot of cell lysate in 0.5 N NaOH was used for determination of protein content by the method of Lowry [Lowry *et al.*, 1951] and in order to determine cell-associated radioactivity, the rest of the cell lysate was mixed with 3 ml of scintillation fluid and kept overnight in the dark before counting in a scintillation counter (Beckman). 2DG uptake was presented as pmol 2DG/mg protein/30 min.

For protein content determination by the method of Lowry [Lowry *et al.*, 1951]; three BSA protein standards of known concentration [0.238 mg/ml; 0.476 mg/ml and 0.952 mg/ml] were used and 0.5 N NaOH used as the blank. The reaction buffer, which contained 2% Na_2CO_3, 1% $CuSO_4.5H_2O$ and 2% NaK^+ tartrate, was freshly prepared prior to experimentation. The assay was done in duplicate and 50 µl of blank, standards and samples were used to perform the protein assay. 1 ml of the reaction buffer was added to the blank, standards and samples, rapidly vortexed and allowed to stand at room temperature for 10 min. Afterwards 0.1 ml Folin-Ciocalteu's phenol reagent (1:2 dilution with distelled water) was added, vortexed and permitted to stand for 30 min. This resulted in a colour development of which the absorbance was read at 750 nm against the blank. The standard curve was used to determine the unknown protein concentrations.

2.7 Pancreatic analysis

The rat pancreata was removed (follows from section 2.2.1 on page 44) and fixed in 4% phosphate buffered formaldehyde whereafter it was processed in an automatic tissue processor through ascending grades of ethanol, xylene and subsequently embedded in paraffin wax. By means of a rotary microtome, the pancreas were sectioned into 4 – 6 mm thick slices, mounted onto glass slides and placed in an oven for 30 min at 60 ^oC. Following the 30 min, each

section was immunolabelled for α-cells with a polyclonal glucagon antibody (Dako, Carpinteria, CA) for 30 min at room temperature. Thereafter, a secondary biotinylated anti-rabbit link antibody was applied and positive labeling was visualised using the peroxidase diaminobenzidine tetrachloride method. This was followed by the immunolabeling of the β-cells, which was accomplished with a monoclonal insulin antibody (Sigma ImmunoChemicals, St. Louis, MO) overnight at 4 ^{o}C. Subsequently, a secondary biotinylated anti-mouse link antibody was utilized and positive labeling was visualised using the alkaline phosphatase fuchsin method. The primary antibody was omitted in the control samples. All sections were counterstained with Mayers haematoxylin for 2 min, air dried and mounted in Entallen®.

The image analysis system which was used comprised of a Leica DC290 digital camera that was mounted on an Olympus BX60 light microscope. The light microscope was interfaced with a personal computer via Leica Qwin® Professional Software. All sections of each pancreas were viewed in entirety with an x10 objective, the field of view captured and digitized to 768 x 1,024 pixels. The system was calibrated for the x10 objective, in the X and Y direction, using a micrometric square with dimensions of 50 microns. All tissue parameters were measured using a Leica Qwin® routine.

Positive staining was identified by colour segmentation, using RGB thresholding. Firstly, the whole section area was identified and measured. Thereafter, the islets were visually identified (different stained colours), interactively demarcated and their total areas determined. Subsequently, α-cell area (brown positive staining) and β-cell area (red positive staining) were identified and the areas measured. All data was exported and analysed using Microsoft Excel. Endocrine to exocrine ratios were calculated by dividing the total islet area measured by the total tissue area measured. The ratio of β-cell to α-cell was calculated by dividing the total β-cell area by the total α-cell area measured. β-cells were sized using the following β-cell area parameters: 0 – 2500 μm^2; 2501 - 7500 μm^2; 7501 - 12500 μm^2; 12501 - 20000 μm^2; and > 20000 μm^2.

2.8 Biochemical analysis

2.8.1 Blood sample collection

After excision of the hearts, blood samples were collected and placed into Eppendorf tubes. The samples were subjected to centrifugation for 30 min at 4 °C and 15000 rpm, serum removed and stored at - 80 °C until biochemical analysis was done.

2.8.2 Serum insulin determination: Radioimmunoassay (RIA)

(Coat-A-Count® Insulin, Diagnostic Products Corporation, LA, USA)

The blood samples collected at the time of sacrifice (refer to section 2.2.1 on page 44 (STZ) and 2.3.1 (DIO) on page 50) were used for serum insulin determination.

The Coat-A-Count Insulin procedure is a solid-phase RIA. ^{125}I-labeled insulin and the insulin in the blood sample, competes for sites on the insulin-specific antibody. This antibody is immobilized to the wall of the polypropylene tubes. Decanting the supernatant from the tubes terminates the competition and isolates the antibody bound fraction of the radiolabeled insulin. By counting the tubes in the gamma-counter, the presence of insulin in the blood sample can be measured. All samples were done in duplictate.

Prior to the commencement of the assay, all the components of the assay were brought to room temperature, as instructed by the manufacturers. Uncoated 12 x 74 mm polypropylene tubes were labeled for total count (T) and non-specific binding (NSB) respectively. Insulin-antibody coated tubes were labeled for standards (refer to Figure 2.5) and serum sample.

200 µL of the zero calibrator A was pipetted into the NSB and A tubes. 200 µL of the remaining calibrator and serum sample were pipetted in the tubes prepared (as indicated in Figure 2.5). 1.0 ml of ^{125}I insulin (refer to Table 2.3

for the WHO International Reference Preparation (IRP) of Insulin) was added to each tube and subsequently vortexed.

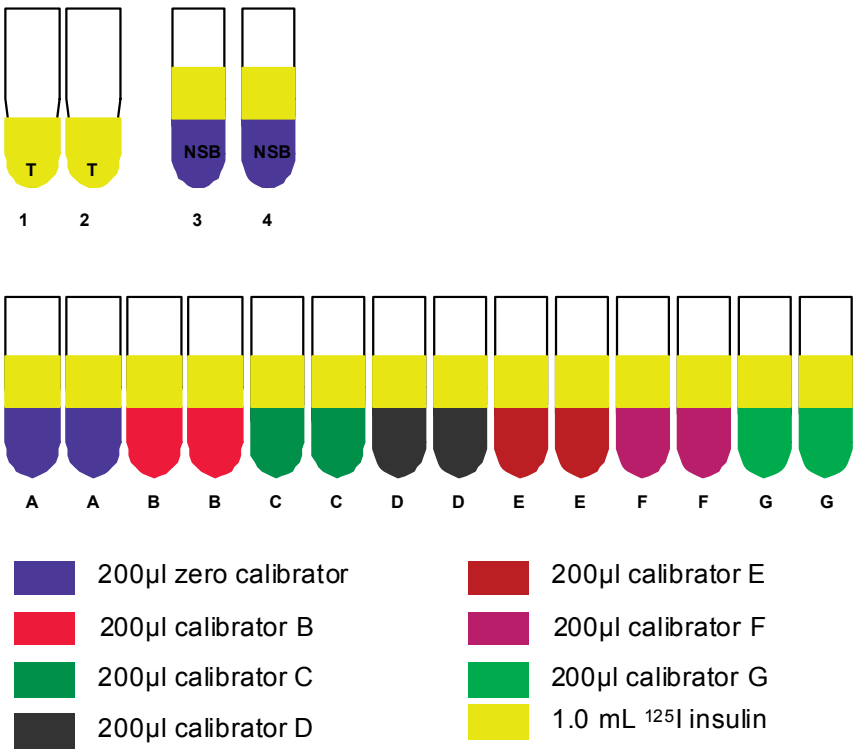
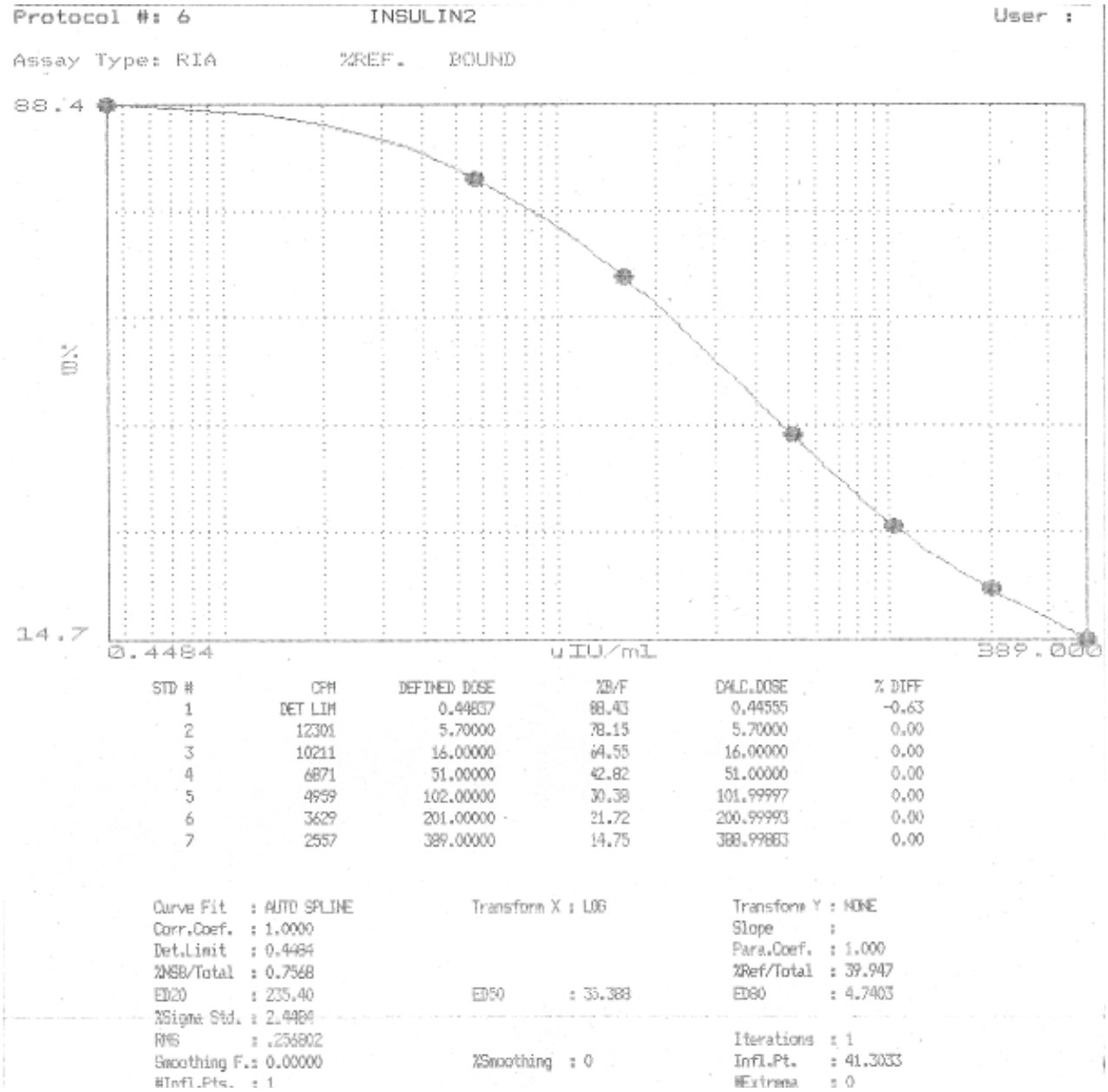


Figure 2.5: Schematic representation of tube preparation

Table 2.3: Tablular representation of the calibrators and WHO International Reference Preparation (IRP) of Insulin (code 66/304) used.

Calibrator	Approximate µIU/ml 1st IRP [code: 66/304]
A	0
B	5
C	15
D	50
E	100
F	200
G	350

Samples were incubated for 18 to 24 hours at room temperature and decanted thoroughly. This was done by placing each tube (except the total count tube) in a foam decanting rack and allowing the tubes to drain for 2 to 3 minutes. Following this, each tube was struck on an absorbent paper and excessive liquid dried from the tubes, to remove the excess moisture for enhanced precision of the assay. The radioactivity of each tube was then measured in a gamma counter (Cobra II Auto Gamma, A. D. P, South Africa) for 1 min per tube and the sample antibody binding affinities, calculated from insulin standard curve, which was generated by the gamma counter. Figure 2.6 is the standard curve generated by the gamma-counter.



STD #	CPM	DEFINED DOSE	%B/F	CALC.DOSE	% DIFF
1	DET LIM	0.44837	88.43	0.44555	-0.63
2	12301	5.70000	78.15	5.70000	0.00
3	10211	16.00000	64.55	16.00000	0.00
4	6871	51.00000	42.82	51.00000	0.00
5	4959	102.00000	30.38	101.99997	0.00
6	3629	201.00000	21.72	200.99993	0.00
7	2557	389.00000	14.75	388.99883	0.00

Curve Fit	: AUTO SPLINE	Transform X	: LOG	Transform Y	: NONE
Corr.Coef.	: 1.0000			Slope	:
Det.Limit	: 0.4484			Para.Coef.	: 1.000
%NSB/Total	: 0.7568			%Ref/Total	: 39.947
ED20	: 235.40	ED50	: 35.388	ED80	: 4.7403
%Sigma Std.	: 2.4484				
RMS	: .256802			Iterations	: 1
Smoothing F.	: 0.00000	%Smoothing	: 0	Infl.Pt.	: 41.3033
#Infl.Pts.	: 1			#Extrema	: 0

Figure 2.6: Standard curve generated by the gamma-counter

2.9 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. Statistical significance between groups was assessed via a 2 way-ANOVA, which was followed by a Bonferroni post-hoc test for multiple comparisons. $p < 0.05$ was considered as statistically significant. Statistical analysis of data was performed using GraphPad Prism 5.

CHAPTER 3

Results: STZ-induced type 1 diabetes rat model

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3. STREPTOZOTOCIN-INDUCED TYPE 1 DIABETES RAT MODEL

STZ is known to have a broad spectrum of properties [Like and Rossini, 1976], of which one is its diabetogenic properties. Its diabetogenic property is characterized by the selective destruction of pancreatic islet β-cells, which ultimately leads to conditions such as insulin deficiency and hyperglycaemia, which mimic human type 1 diabetes mellitus. Several species, including the mouse, rabbit, monkey and the rat (which is our model of choice for this study), are known to be sensitive to the pancreatic β-cell cytotoxic effects of STZ [Wu and Huan, 2008]. Figure 3.1 is a summary of the timeline of the experiment.

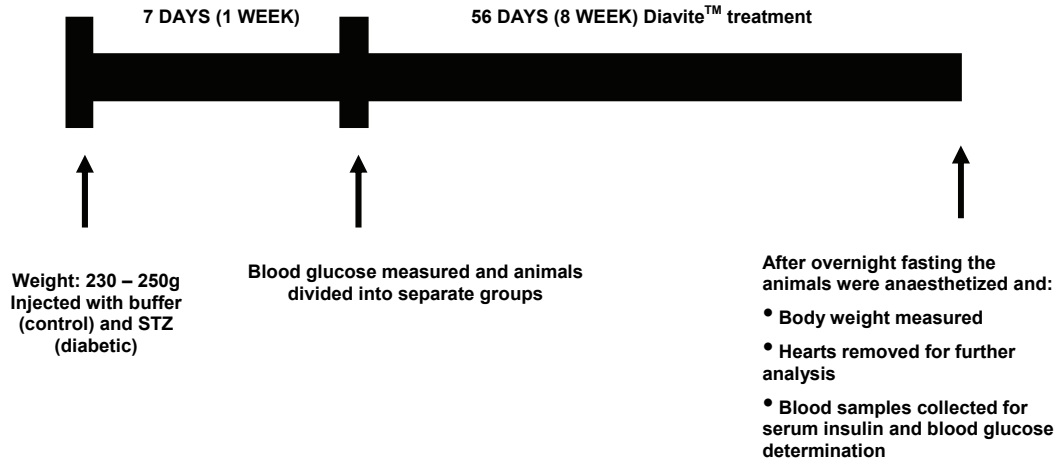


Figure 3.1: Diagram outlining the time-line of STZ experiment

3.1 Characteristics of experimental animals

A single intra-peritoneal injection of 40 mg/kg of STZ markedly decreased body weight ($p < 0.0001$) and increased blood glucose concentrations ($p < 0.001$) when compared to vehicle injected control animals (refer to Table 3). It was found that Diavite™ treatment significantly increased the body weight ($p < 0.001$) and significantly decreased the elevated blood glucose levels of the STZ+Diavite™ animals when compared to the STZ animals ($p < 0.05$). In this study, Diavite™ treatment elicited no significant effect on the body weight and blood glucose levels of the control, non-diabetic animals. Additionally, Diavite™ treatment significantly increased the serum insulin levels of both the control+Diavite™ ($p < 0.001$) and STZ+Diavite™ animals ($p < 0.001$) (refer to Table 3).

Table 3: Weight, fasting blood glucose and serum insulin levels of the experimental animals after a 40 mg/kg STZ injection

Parameters	Control	Control+Diavite™	STZ	STZ+Diavite™
Weight (g)	285.66 ± 19.45	275.82 ± 13.79	241.14 ± 28.55***	260.48 ± 31.03**
Blood glucose (mmol/L)	4.83 ± 0.84	4.90 ± 0.82	19.98 ± 1.31**	15.10 ± 3.42*
Serum insulin level (pmol/L)	328.36 ± 36.74	549.14 ± 73.27**	274.88 ± 31.11	443.09 ± 54.31**

Weight: ***p < 0.0001 STZ vs. control; **p < 0.001 STZ+Diavite™ vs. STZ
Blood glucose: **p < 0.001 STZ vs. control; *p < 0.05 STZ+Diavite™vs. STZ
Serum insulin: **p < 0.001 control+Diavite™ vs. control; STZ+Diavite™ vs. STZ
The data are expressed as mean ± SEM; n = 5 - 8

3.2 Histological analysis

3.2.1 Ratio of β-cell to α-cell area

The dose of 40 mg/kg STZ was sufficient to significantly decrease the ratio of β-cell to α-cell area (0.91 ± 0.22 vs. 1.63 ± 0.41; $p < 0.05$) of the STZ versus control animals (refer to Figure 3.2). This dose of STZ lead to the partial destruction, however, not complete abolishment of the pancreatic β-cell reserve. This is an indication of the succsess of the type 1 diabetes model. Diavite™ treatment tended to increase this ratio in both control+Diavite™ versus control (2.46 ± 0.25 vs. 1.63 ± 0.41) and STZ+Diavite™ versus STZ (1.22 ± 0.42 vs. 0.91 ± 0.22).

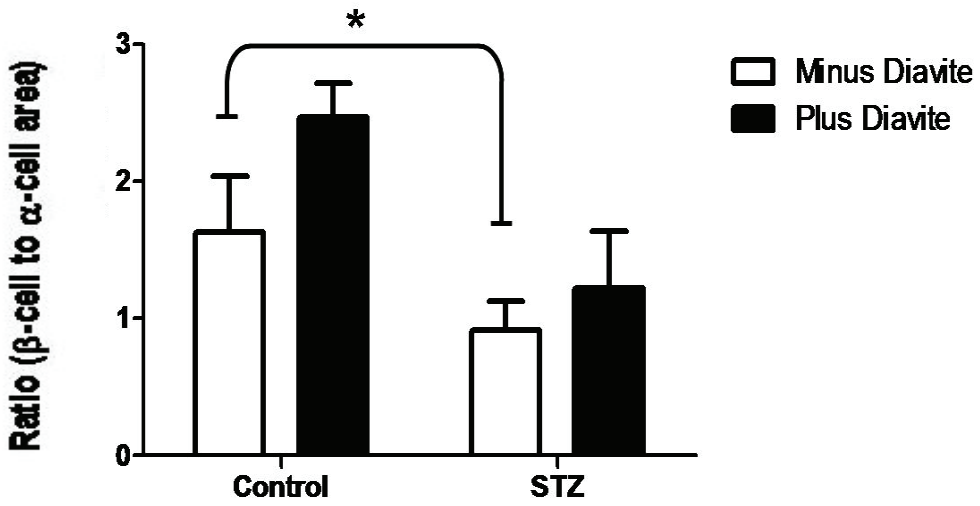


Figure 3.2: Ratio of β-cell to α-cell area. Pancreatic islets were visually identified (different stained colours), interactively demarcated and their total areas determined, thereafter the α-cell area and β-cell area were identified and the areas measured. The ratio of β-cells to α-cells was calculated by dividing the total β-cell area by the total α-cell area measured. The complete description of this method is described in section 2.7 of Materials and Methods. The data are expressed as mean ± SEM.

p < 0.05 control vs. STZ; n = 5 – 8

3.2.2 β-cell regeneration

The STZ group had significantly less small β-cells (0 – 2500 μm^2) (5.43 ± 0.97% vs. 15.33 ± 2.40%; *p < 0.001*), compared to the control group, which was expected since STZ causes β-cell destruction. The effects of Diavite^{TM} as a regenerative agent were observed in the STZ+Diavite^{TM} group. The treatment significantly increased the amount of newly formed small β-cells (10.83 ± 1.45% vs. 5.43 ± 0.97%; *p < 0.01*) within the STZ+Diavite^{TM} group compared to the STZ group (refer to Figure 3.3).

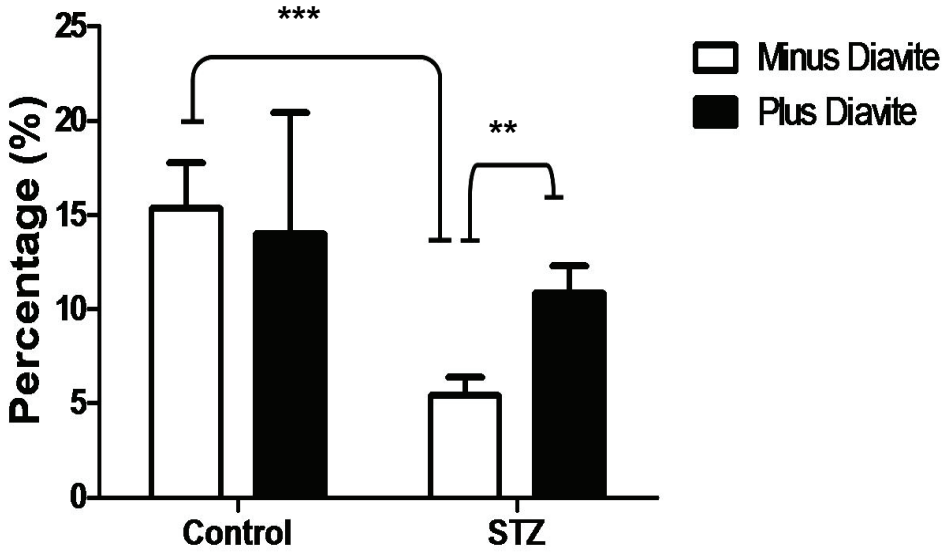


Figure 3.3: Percentage small β-cells (0 – 2500 μm^2). Pancreatic islets were sized using different islet area parameters. The complete description of this method is described in section 2.7 of Materials and Methods. The data are expressed as mean ± SEM.

****p < 0.001 control vs. STZ; **p < 0.01 STZ vs. STZ+Diavite^{TM}; n = 5 – 8***

CHAPTER 4
Results: DIO insulin resistant rat model

4. DIO INSULIN RESISTANT RAT MODEL

The DIO model is a model of hyperphagia induced obesity with the concurrent development of insulin resistance. This model has been characterized in our laboratory and shown to be physiologically relevant and comparable to the human equivalent of insulin resistance as a result of obesity.

The data presented in section 4.1 represents the different end-points at which the experiments were conducted, which is 8, 12 and 16 weeks (refer to section 2.2.2.2 of Materials and Methods). This was done in order to document the progression of obesity in our rat model with regard to certain biometric parameters, in correlation with myocardial damage incurred during ischaemia/reperfusion. The results were obtained through (i) retrograde perfusion experiments, with the end-point of infarct size determination, (ii) isolated cardiomyocyte experiments, with the aim of determining insulin sensitivity and (iii) Western blotting, with the aim of determining protein expression and/or phosphorylation.

4.1 RETROGRADE PERFUSION

Following the 8, 12 and 16 week period on the feeding programme the rats were anaesthetized, their hearts excised and mounted on a Langendorff perfusion apparatus, where after a water-filled latex balloon coupled to a pressure transducer was inserted into the left ventricle of the heart and changes in developed pressure measured. After an ischaemic incident was induced the heart was reperfused and infarct size determined. In sections 4.1.1, 4.1.2 and 4.1.3 follows the characteristics of the 8, 12 and 16-week animals, along with the results of the infarct size measurements.

4.1.1 Animals on 8 weeks feeding programme

4.1.1.1 Characteristics of experimental animals

After 8 weeks on the feeding programme, rats on the high caloric diet (DIO) displayed significantly increased body weight ($p < 0.05$), compared to their age-matched control counterparts (refer to Table 4.1). It was also evident that the group on the DIO diet, presented with increased fed insulin levels, when compared to their control littermates. It is thus apparent that the first signs of metabolic derangements start as early as 8 weeks of high caloric diet consumption (refer to table 4.1).

Table 4.1: Characteristics of the experimental animals after 8 weeks on feeding programme

Parameters	**Control**	**DIO**
Body weight (g)	360.47 ± 11.70	388.54 ± 11.17*
Serum insulin level (pmol/L)	652.69 ± 68.62	823.54 ± 97.51

p < 0.05 control vs. DIO

The data are expressed as mean ± SEM; n = 8

4.1.1.2 Infarct size at 8 weeks

Isolated hearts of DIO animals, after being on an 8 week high caloric diet, presented with a significantly smaller infarcted area (31.05 ± 6.34% of area at risk vs. 47.26 ± 4.10% of area at risk; $p < 0.05$) than control animals, after a regional ischaemic insult (refer to Figure 4.1.1).

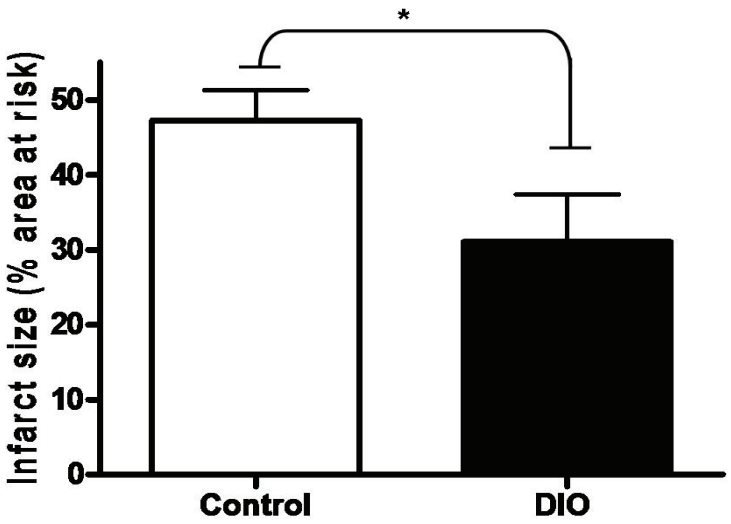


Figure 4.1.1: Infarct size (% area at risk) of control vs. DIO animals after 8 weeks on feeding programme. Hearts were stabilized for 30 min, subjected to 35 min regional ischaemia, followed by 120 min reperfusion, whereafter infarct size was determined. The data are expressed as mean ± SEM.

p < 0.05 control vs. DIO; n = 8

4.1.2 Animals on 12 week feeding programme

4.1.2.1 Characteristics of experimental animals

Contrary to expectations, in this group of animals on the 12 week feeding programme, there were no significant differences in weight gain between the control and DIO animals. However, even without this significant difference in weight gain, it was observe that the DIO animals had significantly higher intra-peritoneal fat mass ($p < 0.0001$) and a significant increase in serum insulin levels ($p < 0.001$), compared to the control animals (refer to Table 4.2). This is an indication that the consumption of a high caloric diet does lead to insulin resistance, even in the absence of total body mass increase. Diavite™ did not have any effect on the total body mass or intra-peritoneal fat mass of the control+Diavite™ versus controls and DIO+Diavite™ versus DIO. However, Diavite™ treatment significantly increased ($p < 0.01$) the already elevated serum insulin levels in the DIO+Diavite™ group when compared to the DIO group (refer to Table 4.2).

Table 4.2: Characteristics of the experimental animals after 12 weeks on feeding programme (8 weeks + 4 weeks Diavite™ treatment)

Parameters	Control	Control+Diavite™	DIO	DIO+Diavite™
Body weight (g)	459.15 ± 15.19	450.02 ± 15.83	457.28 ± 22.23	496.79 ± 16.67
Intra-peritoneal fat (g)	11.19 ± 0.82	9.96 ± 0.55	17.52 ± 1.07***	16.11 ± 1.11***
Serum insulin level (pmol/L)	581.85 ± 54.80	629.56 ± 116.47	747.70 ± 71.39**	1269.75 ± 172.65*

Intra-peritoneal fat: ***p < 0.0001 DIO vs. control; DIO+Diavite™ vs. Control+Diavite™

Serum insulin: **p < 0.001 DIO vs. control; *p < 0.01 DIO+Diavite™ vs. DIO

The data are expressed as mean ± SEM; n = 8

4.1.2.2 Infarct size at 12 weeks

At 12 weeks, the cardiac infarct sizes of the DIO animals tended to be smaller (36.00 ± 4.20% area at risk vs. 44.76 ± 3.90% area at risk), compared to the control group. It was found that the cardiac infarcts of Diavite™ treated animals in the control+Diavite™ group, were significantly smaller (32.61 ± 5.47% area at risk vs. 44.76 ± 3.90% area at risk; $p < 0.05$) compared to the control group (refer to Figure 4.1.2). Thus, Diavite™ treatment leads to cardioprotection amongst control animals at 12 weeks.

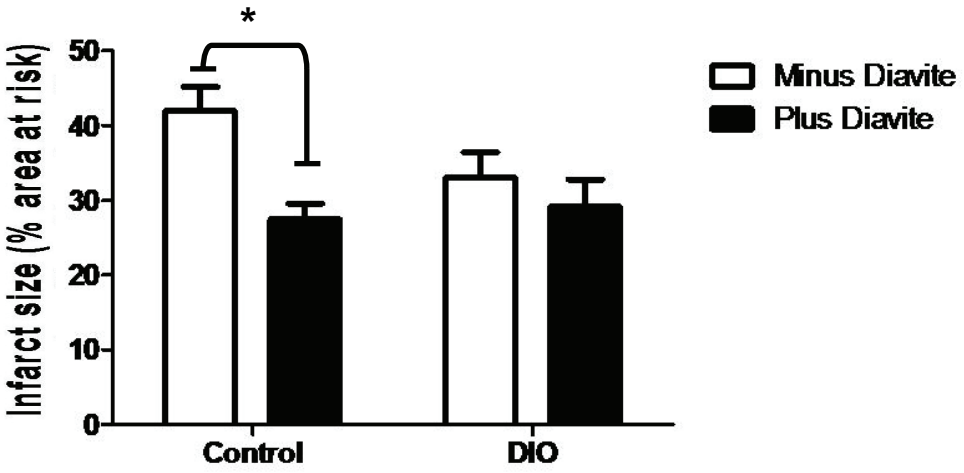


Figure 4.1.2: Infarct size (% area at risk) of control vs. DIO (with and without Diavite™ treatment) animals after 12 weeks on feeding programme. Hearts were stabilized for 30 min, subjected to 35 min regional ischaemia, followed by 120 min reperfusion, whereafter infarct size was determined. The data are expressed as mean ± SEM.

p < 0.05 control vs. control+Diavite™; n = 8

4.1.3 Animals on 16 week feeding programme

4.1.3.1 Characteristics of experimental animals

The DIO group had significantly higher body weights ($p < 0.0001$), compared to their control counterparts, after the 16 week high caloric diet feeding programme (refer to Table 4.3). This obese state was also associated with significant elevation in intra-peritoneal fat mass ($p < 0.0001$), blood glucose ($p < 0.01$) as well as serum insulin levels ($p < 0.0001$). This suggests that our animals developed insulin resistance during this 16 week high caloric feeding programme. Diavite™ treatment was found to further increase the body weight ($p < 0.001$) as well as the serum insulin levels in the DIO+Diavite™ animals ($p < 0.05$), compared to their DIO counterparts (refer to Table 4.3).

Table 4.3: Characteristics of the experimental animals after 16 weeks on feeding programme (8 weeks + 8 week Diavite™ treatment)

Parameters	Control	Control+Diavite™	DIO	DIO+Diavite™
Body weight (g)	456.47 ± 9.74	445.98 ± 11.21	493.82 ± 17.47***	519.42 ± 12.74**
Intra-peritoneal fat (g)	11.60 ± 0.51	10.00 ± 0.43	17.78 ± 1.19 ***	16.05 ± 1.22
Blood glucose (mmol/L)	5.49 ± 0.15	4.98 ± 0.20	6.76 ± 0.29*	6.05 ± 0.76
Serum insulin level (pmol/L)	534.90 ± 29.52	634.98 ± 83.83	759.92 ± 19.03 ***	1016.6 ± 79.31 *#

Body weight: ***p < 0.0001 DIO vs. control; **p < 0.001 DIO+Diavite™ vs. DIO
Intra-peritoneal fat: ***p < 0.0001 DIO vs. control
Blood glucose: *p < 0.01 DIO vs. control
Serum insulin: ***p < 0.0001 DIO vs. control; *p < 0.05 DIO+Diavite™ vs. DIO; #p < 0.001 DIO+Diavite™ vs. control+Diavite™
The data are expressed as mean ± SEM; n = 9

4.1.3.2 Infarct size at 16 weeks

No significant difference was observed in infarct size between the control and DIO animals, after being on the 16 week feeding programme. Diavite™ treatment did, however, significantly decrease the infarcted area of both the control+Diavite™ compared to control animals (36.78 ± 3.11% area at risk vs. 45.11 ± 2.67% area at risk.; $p < 0.05$) and the DIO+Diavite™ compared to DIO animals (36.48 ± 3.18% area at risk vs. 43.08 ± 3.77% area at risk; $p < 0.05$) (refer to Figure 4.1.3). Again, this alludes to the cardioprotective effect of Diavite™ treatment.

Figure 4.1.3: Infarct size (% area at risk) of control vs. DIO (with and without Diavite™ treatment) animals after 16 weeks on feeding programme. Hearts were stabilized for 30 min, subjected to 35 min regional ischaemia, followed by 120 min reperfusion, whereafter infarct size was determined. The data are expressed as mean ± SEM.

p < 0.05 control vs. control+Diavite™; DIO vs. Diavite™; n = 9

4.2 ISOLATED CARDIOMYOCYTES

In this model, cardiomyocytes were prepared from hearts of animals on the 16-week feeding programme (as described in section 2.5 of Materials and Methods). This was done in order to determine the insulin sensitivity of these cardiomyocytes. This is routinely accomplished in our laboratory by means of measuring the ability of the cells to accumulate radio-labeled deoxy-glucose before and after stimulation with insulin (refer to section 2.6 of Materials and Methods).

4.2.1 Basal glucose uptake by cardiomyocytes after 30 min

At basal levels, no significant differences were found between the insulin sensitivity of the cardiomyocytes from the control animals versus cardiomyocytes from the DIO animals. However, Diavite^{TM} treatment did significantly increase the amount of deoxy-glucose accumulation by the cardiomyocytes (18.33 ± 3.59 pmol DG/mg prot/30min vs. 9.38 ± 0.74 pmol DG/mg prot/30min; $p < 0.01$) prepared from the DIO+Diavite^{TM} hearts, which indicates that Diavite^{TM} treatment increases basal glucose uptake by the cardiomyocytes. In addition, analysis by 2-way ANOVA showed the overall effect of Diavite^{TM}, on basal glucose uptake, to be significant ($p < 0.05$).

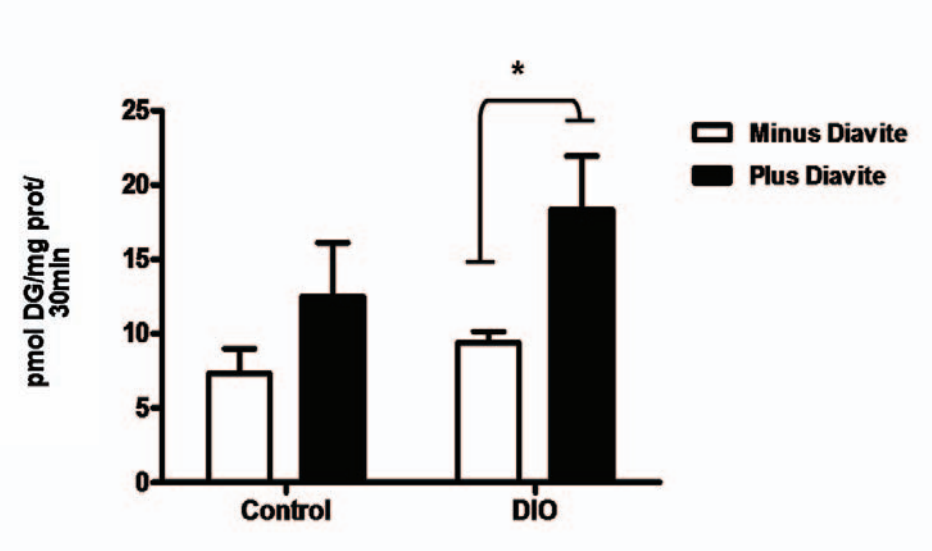


Figure 4.2.1: Basal glucose uptake by cardiomyocytes from control vs. DIO rats after 30 min. The insulin sensitivity of cardiomyocytes was determined by means of measuring the ability of the cells to accumulate radio-labeled deoxy-glucose. Refer to section 2.6 of Materials and Methods. The data are expressed as mean±SEM.

p < 0.01 DIO vs. DIO+Diavite^{TM}; n = 6

4.2.2 Glucose uptake by cells of control animals versus cells of DIO animals after insulin stimulation at different concentrations

4.2.2.1 Stimulation with 1 nM (1000 pmol/L) and 10 nM (10000 pmol/L) insulin

After cardiomyocytes were stimulated with insulin at concentrations of 1 nM (Figure 4.2.2 A) and 10 nM (Figure 4.2.2 B) respectively, Diavite™ treatment tended to increase deoxy-glucose uptake at both concentrations within the DIO group, however the most significant elevation was observed after insulin stimulation at a concentration of 100 nM (Figure 4.2.3).

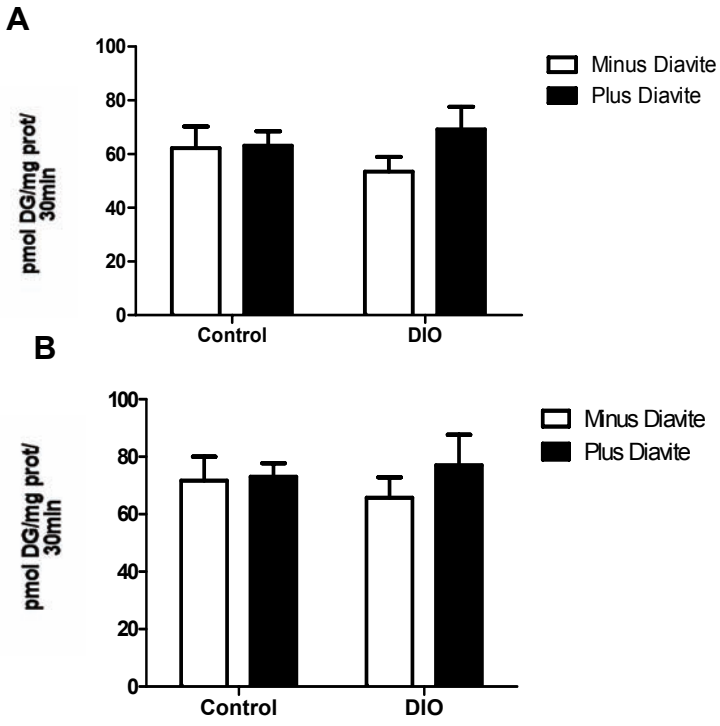


Figure 4.2.2: Glucose uptake by cardiomyocytes from control vs. DIO rats after stimulation with (A) 1 nM and (B) 10 nM insulin. The insulin sensitivity of cardiomyocytes was determined by means of measuring the ability of the cells to accumulate radio-labeled deoxy-glucose after insulin stimulation. Refer to section 2.6 of Materials and Methods. The data are expressed as mean±SEM. n = 6

4.2.2.2 Stimulation with 100 nM (100000 pmol/L) insulin

After stimulation with 100 nM insulin, no significant difference were observed between the insulin sensitivity of cardiomyocytes prepared from control hearts versus cardiomyocytes prepared from DIO hearts. However, Diavite^{TM} treatment resulted in significantly higher glucose uptake by cardiomyocytes prepared from DIO+Diavite^{TM} animals (90.09 ± 13.62 pmol DG/mg prot/30min vs. 65.13 ± 7.33 pmol DG/mg prot/30min; $p < 0.05$) compared to their DIO counterparts, indicating a higher level of insulin sensitivity (refer to Figure 4.2.3). In addition, analysis by 2-way ANOVA showed the overall effect of Diavite^{TM} treatment on glucose uptake after 100 nM insulin stimulation, to be significant ($p < 0.05$).

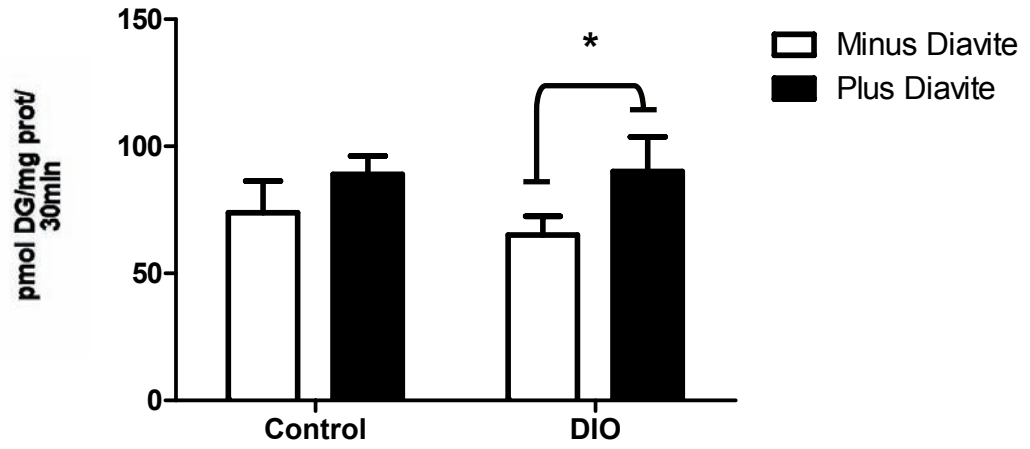


Figure 4.2.3: Glucose uptake by cardiomyocytes from control vs. DIO rats after stimulation with 100 nM insulin. The insulin sensitivity of cardiomyocytes was determined by means of measuring the ability of the cells to accumulate radio-labeled deoxy-glucose after insulin stimulation. Refer to section 2.6 of Materials and Methods. The data are expressed as mean±SEM.

p < 0.05 DIO vs. DIO+Diavite^{TM}; n = 6

4.3 PROTEIN EXPRESSION DETERMINATION (WESTERN BLOTS)

In order to understand and explain the enhanced basal glucose uptake and improved insulin sensitivity found in cardiomyocytes after Diavite^{TM} treatment, we evaluated the expression and activation of key enzymes in the insulin signaling pathway.

Following the 16-week period on the feeding programme the rats were anaesthetized, their hearts excised, freeze-clamped and used for protein expression determination by means of Western blotting techniques (refer to section 2.4 of Materials and Methods). Our aim was to investigate whether Diavite^{TM} treatment had any effect on the expression and/or activation of GLUT1, GLUT4, total IRβ, total and phosphorylated PKB/Akt, total and phosphorylated PTEN and total and phosphorylated PI3K p85.

4.3.1 Myocardial GLUT1 content

It is known that in the heart, GLUT1 protein is associated with basal glucose uptake [Abel, 2004].

No significant difference were found in the GLUT1 content between the different experimental groups, as shown in Figure 4.3.1.

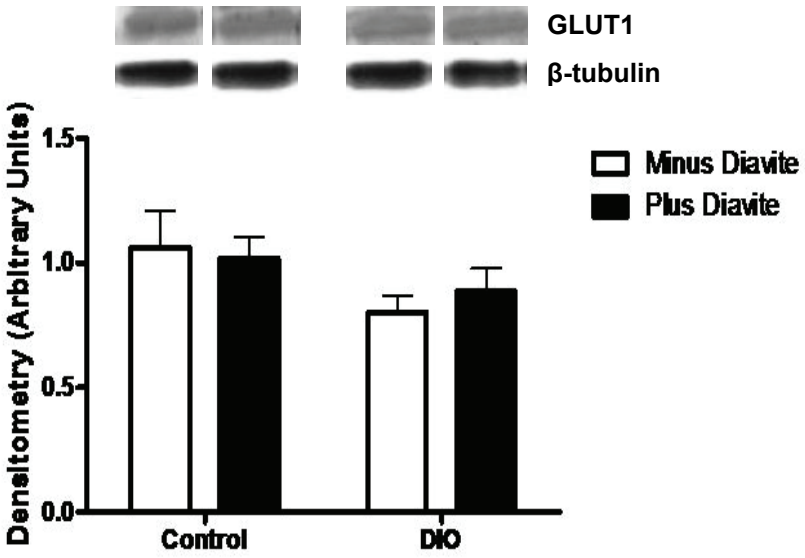


***Figure 4.3.1**: Total protein levels of GLUT1 in hearts from control and DIO animals, with and without Diavite™ treatment. Stripped blots from control and DIO hearts were reprobed with an antibody against β-tubulin to confirm equal loading of the protein. The inserts are representative bands from the actual Western blots. The top band represents GLUT1 and the bottom band represents β-tubulin. The data is presented as mean ± SEM. n = 6*

4.3.2 Myocardial GLUT4 content

GLUT4 has previously been shown to be the main carrier protein associated with insulin stimulated glucose uptake [Watson and Pessin, 2007; Abel, 2004].

There was no significant differences in GLUT4 content between the different experimental groups (refer to Figure 4.3.2).

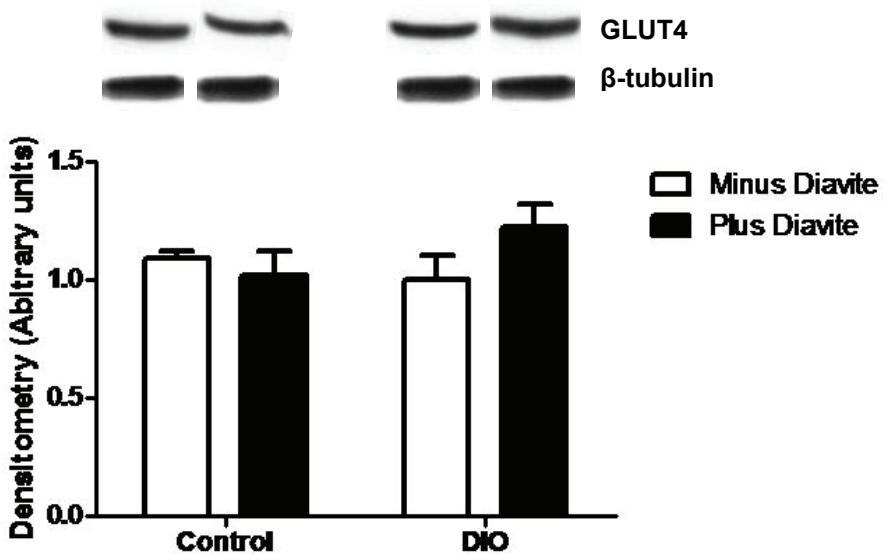


Figure 4.3.2**: **Total protein levels of GLUT4 in hearts from control and DIO animals, with and without Diavite^{TM} treatment. Stripped blots from control and DIO hearts were reprobed with an antibody against β-tubulin to confirm equal loading of the protein. The inserts are representative bands from the actual Western blots. The top band represents GLUT4 and the bottom band represents β-tubulin. The data is presented as mean ± SEM. n = 6

4.3.3 Myocardial total IRβ content

The insulin receptor (IR) is a transmembrane receptor, consisting of two α- and two β-subunits and it is activated by insulin binding [Patti and Kahn, 1998; Van Obberghen *et al.*, 2001; Hubbard, 1997]. The binding of the IR to its downstream proteins eventually leads to the translocation of the glucose transporters to the outer membrane of insulin-responsive tissues, which in turn leads to increased uptake of glucose from blood into these tissues [Huang and Czech, 2007; Watson and Pessin, 2006].

The total expression of IRβ content did not differ significantly between the experimental groups (refer to Figure 4.3.3).

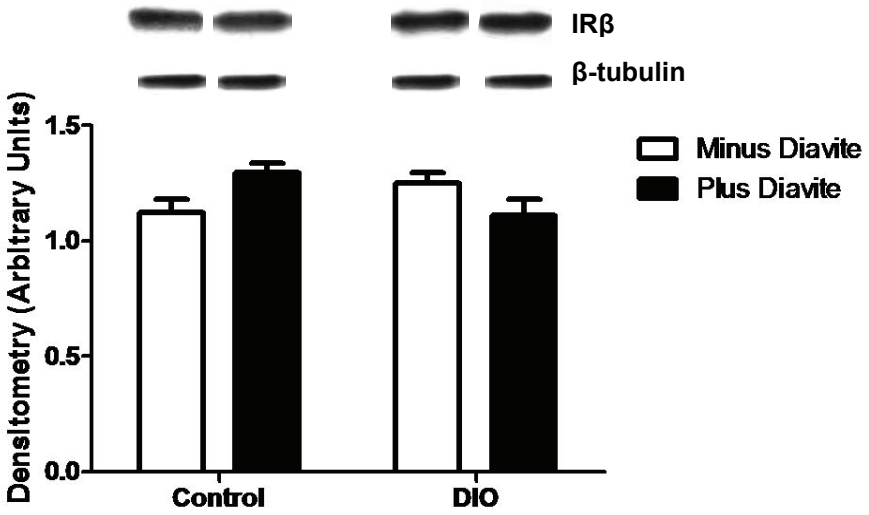


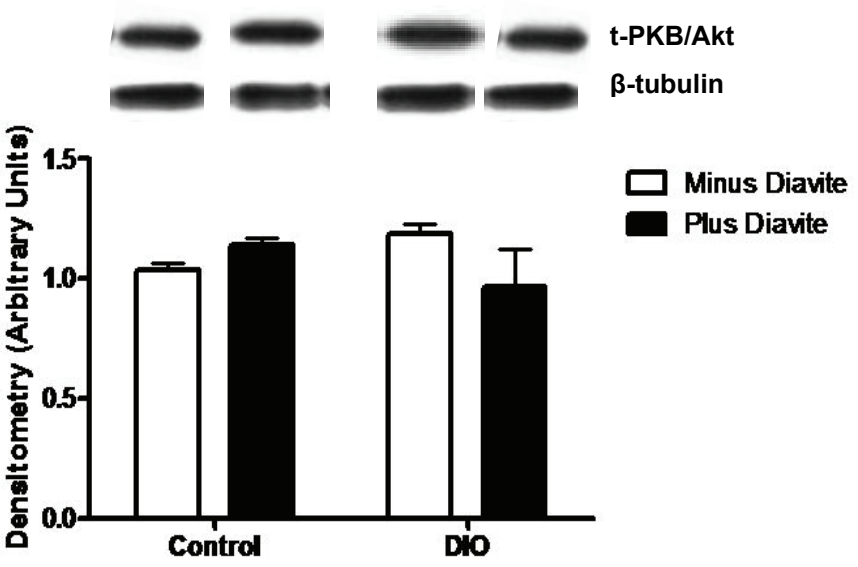
Figure 4.3.3: Total protein levels of IRβ in hearts from control and DIO animals, with and without Diavite™ treatment. Stripped blots from control and DIO hearts were reprobed with an antibody against β-tubulin to confirm equal loading of the protein. The inserts are representative bands from the actual Western blots. The top band represents IRβ and the bottom band represents β-tubulin. The data is presented as mean ± SEM. n = 6

4.3.4 Myocardial PKB/Akt content

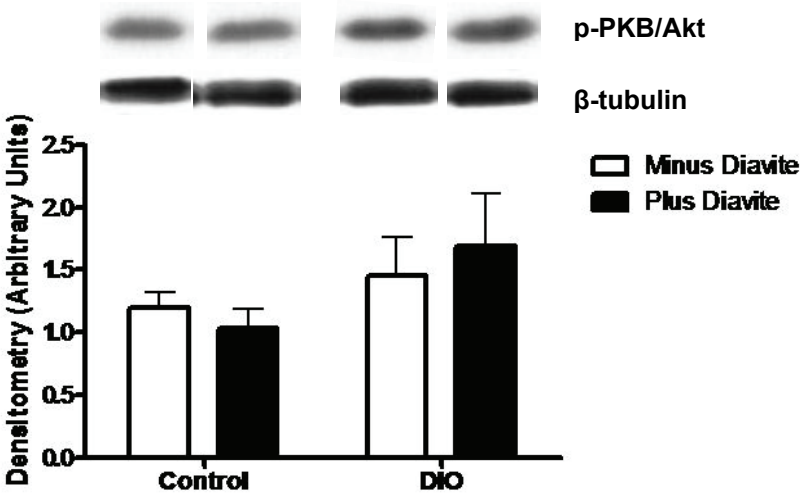
PKB/Akt is a known mediator of the metabolic effects of insulin and it is suggested that activation thereof is involved in processes such as the stimulation of glucose transport and anti-apoptotic processes, which are initiated by insulin [Duronio, 2008; Baines *et al.*, 1999].

Under basal conditions, no significant difference could be found between either the total PKB/Akt or the Ser^{473} phosphorylation of PKB/Akt in our experimental groups (refer to Figure 4.3.5 A and B). However, it was found that the ratio of phosphorylated vs. total PKB/Akt was significantly reduced in the DIO animals (64.88 ± 3.47 AU vs. 99.85 ± 2.08 AU; $p < 0.001$) compared to their control counterparts, thus less total PKB/Akt was phosphorylated in this group (refer to Figure 4.3.4 C). Additionally, a significant reduction was observed in this ratio when control+Diavite™ were compared with control animals (71.53 ± 6.01 AU vs. 99.85 ± 2.08 AU; $p < 0.001$).

A. Total PKB/Akt



B. Phosphorylation of PKB/Akt



C. Ratio of phosphorylated versus total PKB/Akt

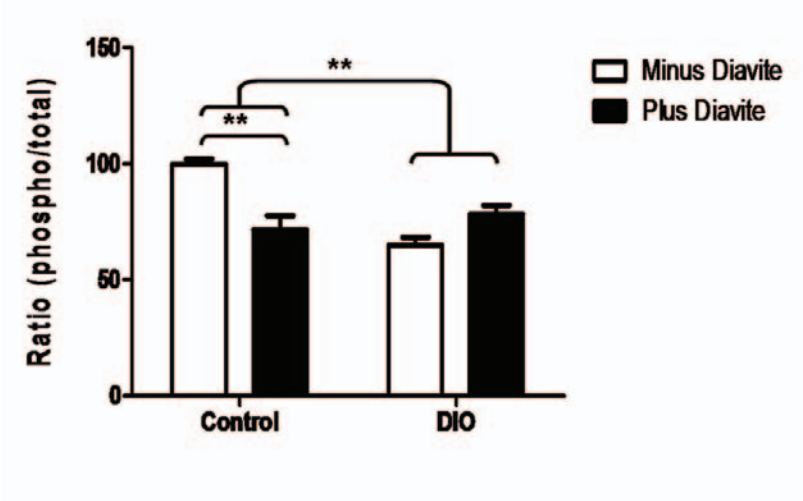


Figure 4.3.4: (A) Total, (B) phosphorylated and (C) ratio of phosphorylated vs. total levels of PKB/Akt in control and DIO animals, with and without Diavite™ treatment. Stripped blots from control and DIO hearts were reprobed with an antibody against β-tubulin to confirm equal loading of the protein. The inserts are representative bands from the actual Western blots. Top band represents PKB/Akt and bottom band represents β-tubulin. Data presented as mean ± SEM.

p = 0.001 control vs. DIO; control vs. control+Diavite™; n = 6

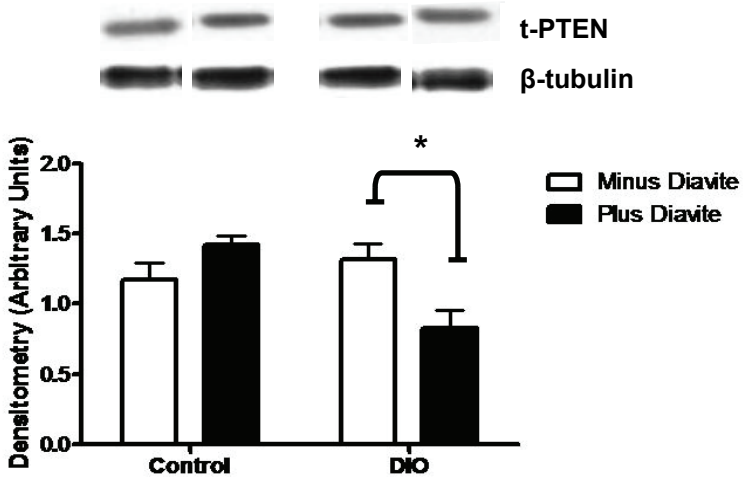
4.3.5 Myocardial PTEN content

The lipid phosphatase PTEN has been implicated as a negative regulator of the PI3K pathway. PTEN dephosphorylates the PI3K product PIP_3 to PIP_2, thereby blocking the cascade of events generated as a consequence of the accumulation of secondary messengers in the plasmalemma. PTEN is thus thought to be the main downregulator of the survival pathway [Hlobilkova *et al.*, 2003; Mocanu and Yellon, 2007].

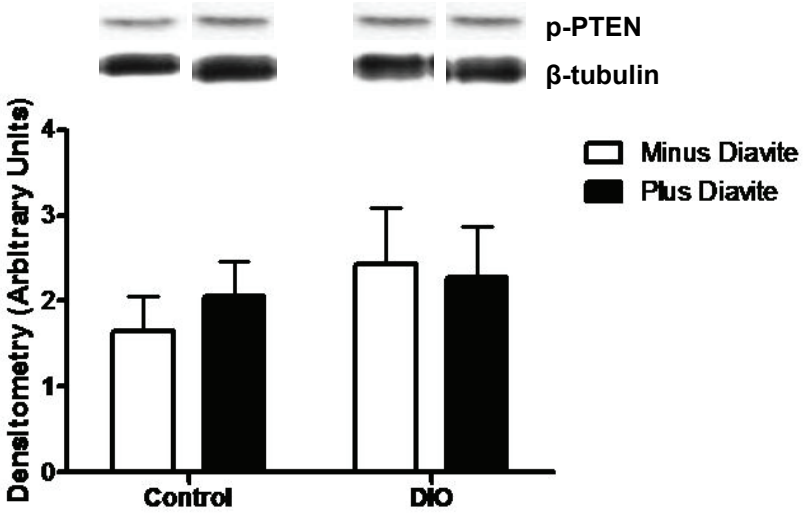
There were no differences observed in the expression of PTEN proteiens at basal level in the hearts, when DIO animals were compared to control animals. However, total PTEN protein levels were significantly suppressed (0.83 ± 0.13 AU vs. 1.32 ± 0.12; $p < 0.01$) in hearts of the DIO+Diavite^{TM} group, compared to DIO animals (refer to Figure 4.3.5 A). As PTEN is a negative regulator of PI3K, suppressed PTEN suggests improved insulin signaling.

The activity of PTEN was evaluated by measuring phosphorylation at the Ser^{380}, Thr^{382} and Thr^{383} sites. Phosphorylation at these sites is associated with inhibition of PTEN activity. No significant differences were found in the phosphorylation state between our experimental groups (refer to Figure 4.3.5 B). However, the calculated ratio of phosphorylated versus total PTEN (Figure 4.3.5 C) indicated a tendency of more total PTEN protein being phosphorylated in the DIO+Diavite^{TM} group compared to the DIO group. If basal phosphorylation of PTEN is upregulated it suggests a decreased PTEN activity, which would allow an increased PKB/Akt phosphorylation with the resultant increased glucose uptake.

A. Total PTEN



B. Phosphorylation of PTEN



C. Ratio of phosphorylated versus total PTEN

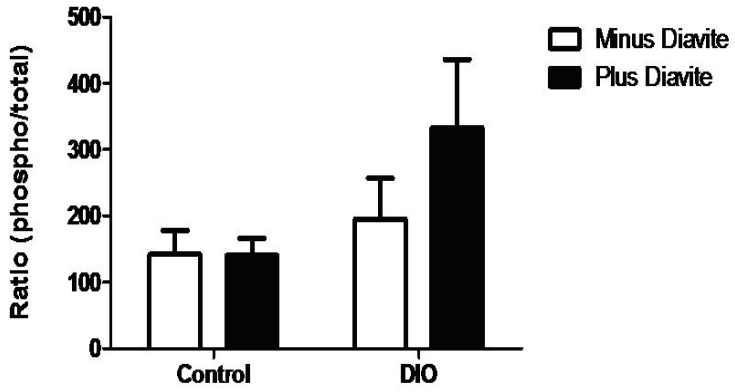


Figure 4.3.5: (A) Total, (B) phosphorylated and (C) ratio of phosphorylated vs. total levels of PTEN in control and DIO animals, with and without Diavite™ treatment. Stripped blots from control and DIO hearts were reprobed with an antibody against β-tubulin to confirm equal loading of the protein. The inserts are representative bands from the actual Western blots. The top band represents PTEN and the bottom band represents β-tubulin. The data is presented as mean ± SEM.

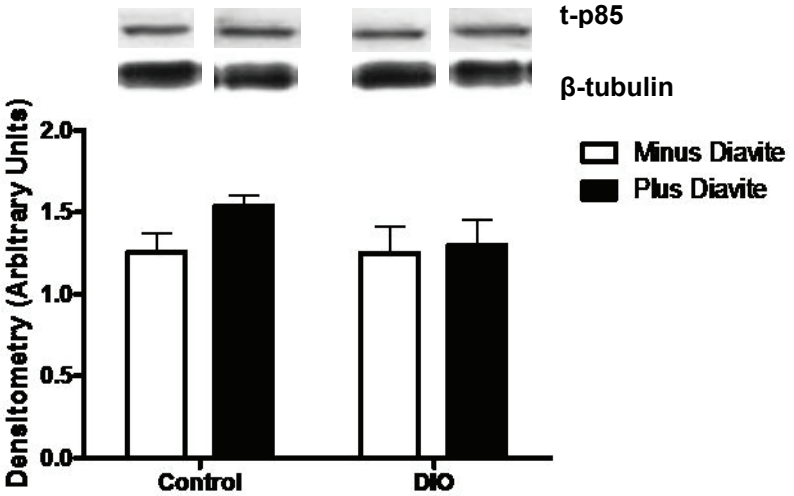
****p < 0.01 DIO vs. DIO+Diavite™; n = 6***

4.3.6 Myocardial p85 content

PI3K is a dimer consisting of a catalytic subunit (p110) and a regulatory subunit (p85). We evaluated the expression and phosphorylation of the regulatory p85 subunit, as phosphorylation of this subunit is associated with activation of the enzyme. PI3K is a key mediator of insulin signaling, thus the enhanced phosphorylation will indicate enhanced signaling via this pathway [Duronio, 2008; Parcellier *et al*., 2008; Barthwal *et al.,* 2003].

No significant differences were observed in the total expression of p85 between the experimental groups (refer to Figure 4.3.6 A), however when measuring the phosphorylated p85, we found that the phosphorylation of p85 was significantly reduced (0.77 ± 0.12 AU vs. 1.18 ± 0.08 AU; $p < 0.0001$) in the DIO group compared to their control counterparts (refer to Figure 4.3.6B). Diavite^{TM} treatment had no additional effect on p85 activity. This would indicate that signaling via PI3K is attenuated in the DIO animal hearts and that Diavite^{TM} treatment could not improve this.

A. Total p85



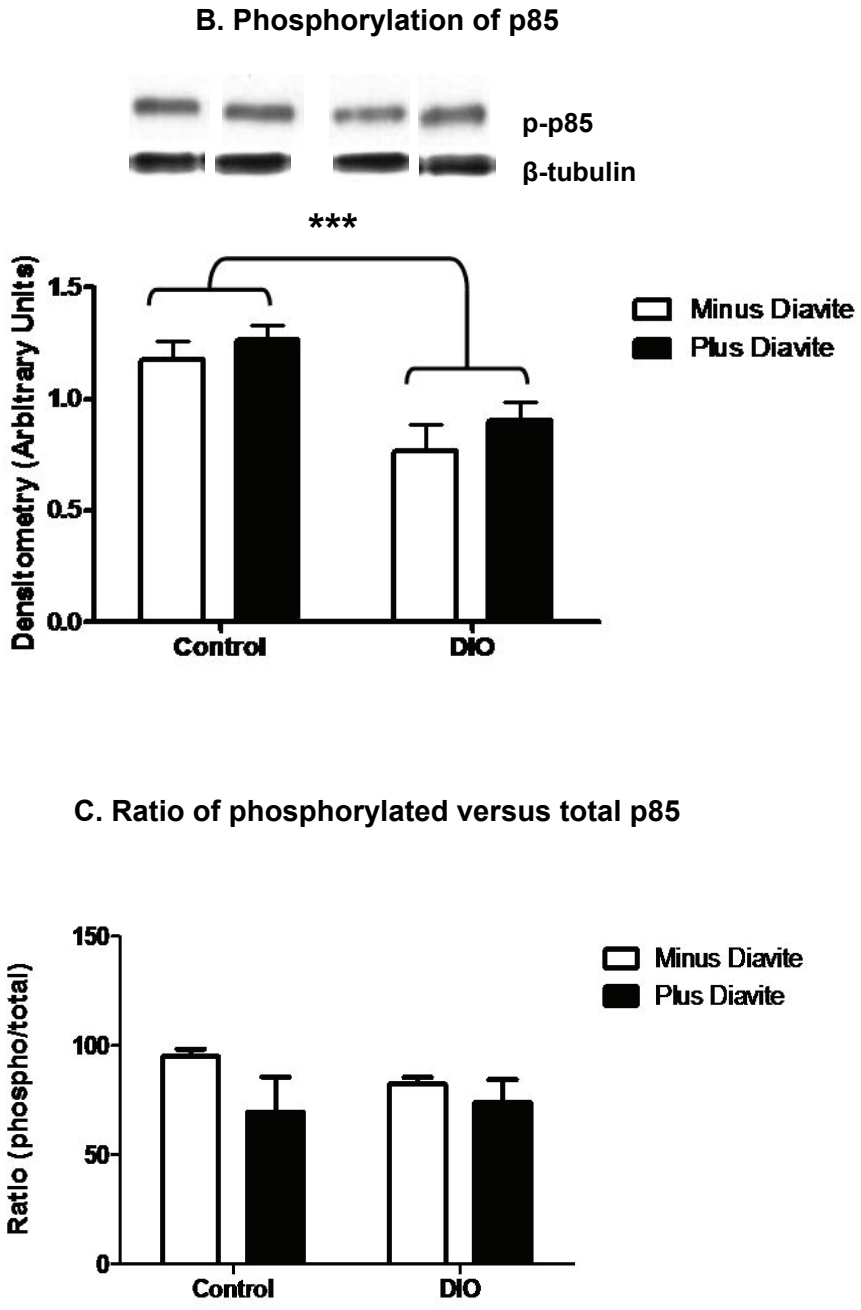


Figure 4.3.6: (A) Total, (B) phosphorylated and (C) ratio of phosphorylated vs. total levels of p85 in control and DIO animals, with and without Diavite™ treatment. Stripped blots from control and DIO hearts were reprobed with an antibody against β-tubulin to confirm equal loading of the protein. The inserts are representative bands from the actual Western blots. Top band represents p85 and bottom band represents β-tubulin. Data presented as mean ± SEM.

****p < 0.0001 control vs. DIO; n = 6***

CHAPTER 5
Discussion

The prevalence of obesity is rampantly increasing and it has focused attention on a worldwide problem [Cannnon and Kumar, 2009; Deitel, 2003; Park *et al.*, 2005; Ogden *et al.*, 2007]. Obesity is a serious public health issue, growing in most countries [WHO, 2006]. Over the past decades, much research has been conducted on the pathophysiology of obesity and its associated complications, such as insulin resistance and the consequent development of type 2 diabetes.

For many years plants have been used as a principal source of medicine for the treatment of various diseases and recently the investigation into plant products have increased. Diavite™ is an herbal product currently marketed as a food supplement with blood glucose and blood pressure stabilizing properties as well as having the ability to enhance glucose utilization. It is manufactured and distributed in South Africa and consists solely of the dried and ground pods of the *P. glandulosa* tree (Honey mesquite). To our knowledge, no studies have been conducted on the mechanisms involved in the effects of this plant product and thus the active component of Diavite™ has not yet been identified. Not many studies have been conducted on the *P. glandulosa* plant itself and we could find no literature on human studies concerning *P. glandulosa*. However, much research has gone into other related species such as *P. juliflora* [Ali *et al.*, 2004; Nakano *et al.*, 2004]. Thus, the mechanisms and effects of this plant and the product manufactured from it are still totally unknown, except for anecdotal claims.

The initial aim of our study was to investigate the claims of anti-diabetic properties made by the producing company. In a pilot study conducted in 2006, it was found that genetically type 2 diabetic rats (Zucker *fa/fa*), treated with Diavite™, had decreased fasting glucose levels and an improved IPGTT when compared to untreated controls. In addition, cardiomyocytes prepared

from these rats, were more insulin sensitive after Diavite^{TM} treatment. The data of this pilot study are not included in this review.

After the promising results from the pilot study, we set out to further investigate the claims of anti-diabetic properties in other animal models as well as investigating the possible mechanisms of action of this product. We accomplished this aim by utilizing two well recognised animal models, namely a (i) STZ type 1 diabetes animal model as well as a (ii) DIO insulin resistant animal model [Arison and Fendale, 1967; Junod *et al.*, 1969; Brondum *et al.*, 2005; Pickavance *et al.*, 1999].

5.1 Streptozotocin-induced type 1 diabetes

Type 1 diabetes is an auto-immune disease, where the β-cells of the pancreas undergo selective destruction. This disease is characterized by hyperglycaemia due to the abolishment of insulin-producing β-cells and is associated with long-term complications, which includes retinopathy, nephropathy, neuropathy and increased risk of cardiovascular disease [Gispen and Biessels, 2000; Vardi *et al.*, 2008].

It has previously been reported that STZ has the ability to produce mild to severe types of diabetes according to the dosages used when it is given to animals either by a single intra-venous or intra-peritoneal injection [Junod *et al.*, 1969]. STZ was initially isolated from *Streptomyces achromogenes* in 1960 and is a known diabetogenic agent for several species, including the rodent [Like and Rossini, 1976]. It is characterized by the selective destruction of pancreatic islet β-cells, hours after STZ injection [Rossini *et al.*, 1977], which ultimately leads to conditions such as insulin deficiency and hyperglycaemia, mimicking human type 1 diabetes mellitus.

5.1.1 Characterization of STZ-induced type 1 diabetes rodent model

For our first model we set out to develop a type 1 diabetes rat model in which the animal still retained a portion of its pancreatic function and not suffer total ablation of its β-cell utilization. We successfully accomplished this with a single intra-peritoneal injection of STZ at a dose of 40 mg/kg. This 40 mg/kg dosage was sufficient in destroying part of the β-cell reserve, which can be observed in the control versus STZ groups (refer to Figure 3.2). A significantly decreased β-cell to α-cell area ratio is observed between these two groups. In addition, fasting blood glucose levels were significantly elevated (refer to Table 3) as early as 2 - 3 days post-injection, which was then followed by a gradual increase in blood glucose levels over the remaining period of experimentation. Within our model a slightly decreased serum insulin level (refer to Table 3) was observed, that would be due to the decreased insulin-producing β-cells (refer to Figure 3.2).

This STZ-induced type 1 diabetes model is the model we utilized to determine Diavite^{TM}'s effects on blood glucose and serum insulin levels as well as its effects on pancreatic β-cells [Akbarzadeh *et al.*, 2007; da Costa and Vianna, 2008].

5.2 DIO insulin resistance

In this study we successfully utilized a model of DIO, which is characterized by hyperphagia, increased thermogenesis, hyperleptinaemia and mild insulin resistance [Pickavance *et al.*, 1999]. This model has been characterized in our laboratory and shown to be physiologically relevant and comparable to the human equivalent of insulin resistance as a result of obesity [Du Toit *et al.*, 2005]. In the DIO model, the diet of the adult rats was changed similarly to the changes experienced by humans, changing from a more frugal, rural diet to a Westernized, fast food diet.

5.2.1 Characteristics of DIO insulin resistant rodent model

Our second animal model was insulin resistance as a result of obesity. Adult male Wistar rats were placed on a high caloric diet for 8, 12 or 16 weeks. This was done in order to document the progression of obesity in our rat model with regard to certain biometric parameters.

After being on the high caloric diet for 8 weeks the DIO animals gained significantly more body weight than controls. However, they did not display signs of insulin resistance (refer to Table 4.1), as there was no significant difference between the serum insulin levels of the control versus DIO animals. After 12 weeks on the high caloric diet the DIO animals in this particular group did not present with increased weight gain compared to their controls as would be expected (refer to Table 4.1.2). However, there was a significant increase in intra-peritoneal fat mass and serum insulin levels (refer to Table 4.1.2), indicative of insulin resistance. This same occurrence has previously been documented by Naderali and colleagues in a paper published in 2001. They found that in a group of rodents that were voluntary fed a high caloric diet for a period of 12 weeks, there were animals that gained significant weight, but that there were also animals that did not gain much weight. Nevertheless, even in those animals that did not gain excessive weight, the diet significantly increased visceral fat mass and plasma triglyceride levels compared to their chow-fed counterparts and that this low weight gain group developed central obesity. This indicates that the consumption of a high caloric diet can lead to early signs of insulin resistance, even in the absence of obesity. Obesity, physical inactivity or genetic predisposition is usually the root for the development of insulin resistance, however, it is not always the case. It has been reported that it is rather the distribution of fat, whether central/visceral or peripheral obesity, that plays a significant role in the development of insulin resistance. Insulin resistance can thus occur in the absence of obesity [Rosenthal *et al.*, 1983]. Following a 16-week feeding program, we successfully generated a model of insulin resistance as a result of obesity, which was the model utilized in the cardiomyocyte and protein expression determination experiments. These animals had significantly

increased weight gain and intra-peritoneal fat mass, which was accompanied by elevated glucose levels and hyperinsulinaemia, which are all trademarks of insulin resistance (refer to Table 4.1.3).

5.3 Effects of Diavite™ consumption

5.3.1 Diavite™ as a β-cell regenerative agent

It is well known that the adult β-cell mass is not static, but that it fluctuates in response to different physiological conditions, such as insulin resistance and type 2 diabetes. This change in β-cell mass accentuates the importance of adaptive mechanisms employed by the pancreas [Bonner-Weir, 2000 (b)]. Methods, such as pancreatectomy or partial duct ligation, were found to initiate these regenerative programmes, which act to replace missing cells [Bonner-Weir *et al.*, 2008]. However, the origin of these regenerative β-cells remains unclear and is an ongoing debate amongst researchers.

By utilizing pulse-chase labeling, Dor and colleagues (2004) established that, throughout adult life or after partial pancreatectomy, new β-cells arose from pre-existing β-cells, rather than from any other cell subtype. Nir and colleagues (2007) obtained similar results after the diphtheria-toxin-mediated ablation of β-cell mass. They found that treated mice recovered from hyperglycaemia through the formation of new β-cells that mainly originated from surviving β-cells. However, numerous other studies suggest alternative origins for β-cells during pancreas regeneration, thereby encouraging the pancreatic stem cell hypothesis [Zulewski *et al.*, 2001; Petropavlovskaia and Rosenberg, 2002]. Wang and colleagues (1995) provided initial evidence of non-β-cells converting to β-cells. In their study, they found that by provoking a regenerative response in rats, through partial duct ligation combined with BrdU pulse-chase labeling, proliferation could not fully account for the drastic β-cell hyperplasia observed [Wang *et al.*, 1995]. These findings thus suggest that multipotent precursors and/or stem cells may contribe to the regenerative capability of the pancreatic islets observed in their study. Xu and co-workers

(2008) confirmed the hypothesis that precursor cells are involved in the β-cell regenerative process. In their study, they found that duct-lining precursor cells were responsible for the pancreatic islet regeneration.

The observation that β-cells can be regenerated by replication of pre-existing β-cells or through the conversion of exocrine cells to β-cells opens new avenues for diabetes treatment.

In our study it was found that Diavite^{TM} treatment tended to increase the β-cell to α-cell area ratio in both control+Diavite^{TM} versus control and STZ+Diavite^{TM} versus STZ (refer to Figure 3.1) groups. Additionally, Diavite^{TM} treatment significantly increased newly formed small β-cells (0 – 2500 μm^2) in the STZ+Diavite^{TM} versus STZ group (refer to Figure 3.2). There were no significant differences found regarding new small β-cells formation in our control+Diavite^{TM} versus control group. This could be because the control group did not undergo β-cell ablation and thus β-cell regeneration would not be necessary.

From this study it is apparent that the consumption of Diavite^{TM} leads to increased β-cells (when comparing β-cell to α-cell area ratio) and Diavite^{TM} treatment increased the formation of small β-cells in the STZ group that suffered β-cell ablation (refer to Figure 3.2). The origin of these newly formed β-cells, whether it is from pre-existing β-cells or from converted exocrine cells, is still unknown and would warrant further investigation. According to Zhou and colluegues (2008), a combination of the transcription factors Pdx1, Ngn3 and MafA is required in the differentiation of endocrine progenitors to hormone-producing β-cells [Zhou *et al.*, 2008]. By determining the expression of these transcription factors (Pdx1, Ngn3 and MafA) in the pancreata of Diavite^{TM} treated animals it can be determined whether these newly formed cells are from transdifferentiated adult pancreatic cells.

The β-cell regenerative capacity of Diavite^{TM} treatment leads to increased β-cell formation with a possible increase in insulin production. This finding is deemed as clinically relevant because being able to enlarge the “window”

period for sufferers of type 1 and most probably type 2 diabetes will lessen β-cell loss for an extended period.

5.3.2 The effect of Diavite^{TM} on insulin secretion

Insulin is the hormone, secreted by the pancreas, which is responsible for glucose uptake and transport to the majority of cells. In response to hyperglycaemia the β-cells secrete insulin, which in turn stimulates the uptake of glucose by peripheral insulin sensitive tissues.

We observed a significant increase in insulin secretion post-Diavite^{TM} treatment in both the STZ-induced type 1 diabetes and DIO insulin resistant rat models. In the STZ-induced type 1 diabetes model, Diavite^{TM} consumption for 8 weeks resulted in significantly increased serum insulin levels in the control as well as the STZ animals (refer to Table 3). This significant increase in serum insulin levels was not accompanied by a concomitant decrease in glucose levels in the control group (refer to Table 3). In the STZ portion of the study, Diavite^{TM} treatment did significantly lower the elevated blood glucose levels, however, the glucose levels were still approximately 3-fold higher than in the control animals. In the DIO insulin resistant model, after 16 weeks on the high caloric diet, Diavite^{TM} treatment significantly increased the insulin levels of the DIO+Diavite^{TM} animals, without any significant decrease in glucose levels. These results suggest that Diavite^{TM} might induce insulin resistance, since the increased insulin levels are not accompanied by a significant reduction in glucose levels. However, cardiomyocytes prepared from animals from the same model (DIO for 16 weeks) accumulated more deoxy-glucose before and after insulin stimulation (refer to Figures 4.2.1 and 4.2.3) compared to their control counterparts after Diavite^{TM} treatment. This increased insulin sensitivity after Diavite^{TM} treatment was also observed in the pilot study, conducted in 2006 (refer to page XXV). In this model, cardiomyocytes prepared from Zucker rats were more insulin sensitive after Diavite^{TM} treatment. Further studies are being conducted to clarify why the

significant increase in insulin levels were not accompanied by a significant reduction in glucose levels in this rodent model of DIO.

5.3.3 The effect of Diavite^{TM} on insulin sensitivity

Obesity has always been linked to insulin resistance or decreased insulin sensitivity and the consequent development of type 2 diabetes [DeFronzo, 1997]. Insulin resistance occurs when there is a defect in insulin action, which inevitably leads to hyperinsulinaemia to maintain euglycaemia. Even though the precise mechanism of insulin resistance remains unclear, the general assumption has always been that insulin resistance is acquired as a result of elevated free fatty acids, which is accompanied by hyperglycaemia and hyperinsulinaemia [DeFronzo, 1997; DeFronzo and Ferrannini, 1991]. Insulin resistance is associated with abnormalities in insulin signal transduction [Haring and Mehnert, 1993; Nolan *et al.*, 1994] and glucose transport. Most investigators have interpreted the hyperinsulinaemia to represent a compensatory response to counteract the impairment in insulin action [DeFronzo, 1997; Reaven, 1988].

Research suggests that more than one molecular defect in the insulin signaling pathway can lead to an insulin resistant phenotype [Woods *et al.*, 2009]. For this reason, we selected and evaluated key proteins that are known to be involved in the insulin signaling pathway and that might be affected during insulin resistance, namely, GLUT1, GLUT4, total IRβ, total and phosphorylated PKB/Akt, total and phosphorylated PTEN and total and phsophorylated PI3K p85. We also evaluated the effect Diavite^{TM} might have on the expression and/or phosphorylation of the above mentioned proteins. These are but a few of the numerous proteins involved in the insulin-signaling cascade.

Refer back to section 2.2 of the literature review for a brief reminder as to how all these proteins fit into the cascade initiated by insulin binding.

5.3.3.1 Myocardial GLUT 1 content

Compared to GLUT4, GLUT1 is the less abundant glucose transporter in the heart and it is known to be responsible for basal glucose uptake [Abel, 2004], thus glucose transport via this transporter is insulin independent.

From the literature is not clear how the expression of GLUT1 is altered during disease states such as obesity and early insulin resistance, however, it is known that GLUT1 expression is augmented during type 2 diabetes. This is thought to be, in part, as a result of prolonged exposure to ROS. The increased flux of free fatty acids and glucose is associated with increased mitochondrial ROS production, which affects transcription of glucose transporters [Bloch-Damti and Bashan, 2005]. Our current model is a model of early insulin resistance or pre-diabetes and not full-blown type 2 diabetes, as indicated by the elevation in glucose levels, but which are still well below the classified level of diabetes (> 8 mmol/L) and the elevated insulin levels (refer to Table 4.3).

In view of the elevated basal glucose uptake by the isolated adult cardiomyocytes, which was observed in our Diavite^{TM} treated animals (see Figure 4.2.1); we investigated whether GLUT1 expression was upregulated. We accomplished this by measuring the total expression of the GLUT1 protein, by means of Western blotting techniques. Even with a significantly elevated basal glucose uptake, we could find no significant difference between the control and DIO animals, with or without Diavite^{TM} treatment (refer to Figure 4.3.1).

Although we did not find GLUT1 to play a significant role in the increased basal glucose uptake, it is possible that enhanced expression of other cardiac glucose transporters contributed to the observed increase in basal glucose uptake. Other glucose transporters and enzymes have been found to contribute to glucose uptake by cardiac muscle, such as the expression of GLUT8. Although this glucose transporter's expression in the heart is much less than in other tissue such as testes, the high efficiency of this glucose transporter suggests that it may play a role in glucose uptake in the heart

[Doege *et al.*, 2000]. We did not, however, measure GLUT8 expression in the heart.

In addition, it is also possible that more GLUT4 is situated in the sarcolemma [Matsui *et al.*, 2006], resulting in higher basal glucose uptake, as it is known that GLUT4 is not only located in intracellular storage compartments, but also at the sarcolemma [Zorzano *et al.*, 1997]. However, in this study, translocation between the plasma membrane and intracellular stores were not evaluated.

5.3.3.2 Myocardial GLUT4 content

GLUT4 is the insulin-responsive glucose transporter [Watson and Pessin, 2007; Abel, 2004] and it is the most abundant glucose transporter in the heart of which its translocation to the plasma membrane plays a vital role in the uptake of glucose into the cell. Along with GLUT1, GLUT4 is also located at the sarcolemma and in intracellular storage compartments [Zorzano *et al.*, 1997]. Elevated free fatty acids and intracellular lipid, observed during obesity, appears to inhibit insulin signaling, leading to a reduction in insulin-stimulated muscle glucose transport that may be mediated by a decrease in GLUT4 translocation [Boden and Shulman, 2002].

In our model we evaluated total GLUT4 protein expression in the control and DIO groups, with and without Diavite^{TM} and not the GLUT4 that translocates to the sarcolemma. We could find no significant differences in total expression of GLUT4 between these groups. As it is known that GLUT4 translocates to the membrane to facilitate with glucose uptake, Diavite^{TM} treatment might facilitates this translocation process of GLUT4. However, we did not measure translocated GLUT4.

5.3.3.3 Myocardial insulin receptor β (IRβ) content

The IR belongs to a large subfamily of receptor tyrosine kinases [Patti and Kahn, 1998], consisting of two extracellular α- and two transmembrane β-subunits. The binding of insulin to the α-subunit induces a rapid conformational change, resulting in the autophosphorylation of specific tyrosine residues of the intracellular region of the β-subunit through a transphosphorylation mechanism [Van Obberghen *et al.*, 2001; Hubbard, 1997].

There is not much literature on the total IRβ expression, but more on the tyrosine activity of this receptor under pathological conditions, such as obesity and insulin resistance [Hotamisligil *et al.*, 1994; Issad *et al.*, 2002; Amer *et al.*, 1987]. It might be that there is no significant difference between the total expression of the IR in obesity and early forms of insulin resistance, compared to controls, as we too observed in our study. Via Western blotting techniques of whole heart tissue samples, we measured the total IRβ expression comparing control with DIO animals, with or without Diavite^{TM} treatment, but could find no significant differences in the total expression of IRβ. Diavite^{TM} treatment did not result in upregulation of myocardial insulin receptors and the elevated glucose uptake, which we observed in the isolated cardiomyocytes, can therefore not be as the result of a higher level of IR expression.

Future studies will warrant investigation into the tyrosine kinase activity of the receptor. It has been observed that in disease states such as obesity and insulin resistance, the insulin receptor (IR) tyrosine kinase activity is defective [Hotamisligil *et al.*, 1994]. In these pathological states insulin's effects on its target tissue are markedly reduced and thus insulin resistance is often associated with a decrease in the tyrosine kinase activity of the IR, resulting in alterations in insulin signaling [Issad *et al.*, 2002]. A study done by Amer and colleagues (1987) on obese, insulin resistant and type 2 diabetic males, they found that the tyrosine kinase activity of the insulin receptor from all insulin resistant groups was 40% less when compared to the activity of lean control subjects. This alteration was present in the absence of changes in the level of

the insulin receptor or its insulin binding characteristics. For the purpose of our study we did not measure the activity of the IR.

5.3.3.4 Myocardial PKB/Akt content

PKB/Akt is an important downstream kinase of post-insulin-receptor signaling and has been reported to be a good reflection of insulin's activity. PKB/Akt is known as a mediator of the metabolic effects of insulin and it is suggested that activation thereof is involved in numerous processes, such as the stimulation of glucose transport and anti-apoptotic processes (cardioprotection), initiated by insulin [Opie, 1998]. PKBβ/Akt2 (one of the PKB/Akt isoforms) knockout mice were found to develop severe diabetes. In the absence of PKB*β*/Akt2, insulin's action was blocked and the mice were insulin resistant, with hyperglycaemia, hyperinsulinaemia and glucose intolerance [Cho *et al.*, 2001]. These observations suggest that PKB*β*/Akt2 plays a critical role in glucose metabolism. In insulin resistance, dysregulation of PKB/Akt activation may result in abnormalities in insulin signaling, causing attenuated glucose transport response.

It has been established that the activation of the PI3K/PKB/Akt signaling pathway is also essential for protection against ischaemia/reperfusion injury [Mocanu and Yellon, 2007; Hausenloy and Yellon, 2004]. We proposed that increased PKB/Akt phosphorylation could be responsible for the reduced infarct size observed after Diavite™ treatment when comparing the control+Diavite™ to the control group as well as the DIO+Diavite™ to the DIO group at 16 weeks. However, this was not the case, as we could find no significant difference in phosphorylated PKB/Akt (refer to Figure 4.3.4 B). There was also no significant differences in total PKB/Akt expression between the experimental groups (refer to Figure 4.3.4 A). These results indicate that the altered basal levels of PKB/Akt expression or phosphorylation may not have played a role in the cardioprotection that resulted in the reduced infarct size we observed, after Diavite™ treatment. Although the ratio of phosphorylation versus total PKB/Akt was lower in the DIO group, Diavite™

treatment did not change this. In this study, we did not measure PKB/Akt phosphorylation after insulin stimulation or after ischaemia/reperfusion (as was done for infarct size determination) of the heart, both of which may differ after Diavite^{TM} treatment.

5.3.3.5 Myocardial PTEN content

PTEN has been implicated as a negative regulator of the PI3K pathway [Sasaoka *et al.*, 2006; Mocanu and Yellon, 2007; Hlobilkova *et al.*, 2003]. PTEN dephosphorylates the PI3K product PIP_3, which serves as crucial lipid second messenger in various metabolic effects of insulin, to PIP_2, thereby reducing PKB/Akt activity. PTEN is thus thought to be the main downregulator of this survival pathway [Hlobilkova *et al.*, 2003; Mocanu and Yellon, 2007]. PTEN is ubiquitously present in normal cells and its activity can be downregulated by phosphorylation or oxidation. The opinion is that in its phosphorylated state, PTEN is inactive [Vazquez *et al.*, 2000]. PTEN can also be inactivated through oxidation induced by ROS [Leslie *et al.*, 2003]. This seems to be the main process, in the acute setting, that regulates PTEN's activity. Amongst the metabolites that induce transcription of PTEN are PPAR-γ [Teresi *et al.*, 2006] and the tumor suppressor p53 [Wang *et al.*, 2005]. The regulation of PTEN and its activity is complex and is yet not completely understood, but from what is known, PTEN seems to be an important "switch" in the aim of maintaining cellular homeostasis and normal development.

In our study, we found that the total expression and activity of PTEN was decreased in the DIO+Diavite^{TM} group when compared to the DIO group (refer to Figure 4.3.6 A). Suppressed PTEN suggests alleviation in insulin resistance. We found no significant differences in the phosphorylated PTEN between these different groups (refer to Figure 4.3.6 B). However, it seems as if more of the total PTEN protein is phosphorylated in the DIO+Diavite^{TM} when compared to the DIO group (refer to Figure 4.3.4 C). If basal phosphorylation of PTEN is upregulated it suggests a decreased PTEN activity, which would allow an increased PKB/Akt phosphorylation with the resultant increased

glucose uptake. This result is of clinical importance because PTEN phosphorylation leads to the activity of PTEN to be suppressed, which consequently indicate an increased activity of PKB/Akt and increased cellular insulin sensitivity.

5.3.3.6 Myocardial p85 content

It is well recognised that PI3K is as an essential signal transduction element in most the cellular processes, regulated by insulin [Alessi and Downes, 1998]. In addition, PI3K regulates multiple and diverse cellular functions, including insulin-stimulated glucose transport and cellular survival. PI3K consists of a catalytic subunit, p110 and a regulatory subunit, p85 [Alessi and Downes, 1998]. The regulatory subunit, p85, interacts with tyrosine-phosphorylated motifs via two SH2 domains, finally leading to an increased catalytic activity of the PI3K complex [Myers *et al.*, 1992].

In a study done by Cusi and co-workers (2000) it was found that obese non-diabetic patients showed reduced stimulation of the PI3K pathway, indicating the presence of insulin resistance. Our findings, therefore, corroborate that insulin resistance is accompanied by attenuated signaling through PI3K as evidenced by suppressed p85 phosphorylation in our DIO group. Diavite^{TM} treatment did not improve this.

Our laboratory studies have shown that Diavite^{TM} has an effect on insulin sensitivity. We found that in our DIO insulin resistant model, Diavite^{TM} consumption significantly increased glucose uptake by cardiomyocytes, at basal levels as well as with insulin stimulation, which is an indication of increased insulin sensitivity. Diavite^{TM} had effectively improved insulin sensitivity in DIO cardiomyocytes, however, not in control cardiomyocytes. The above results regarding the protein expression, was conducted at basal conditions, thus without insulin stimulation, which can be the reason for us not observing increased p85 phosphorylation after Diavite^{TM} treatment. Our

results also indicate that neither elevated expression nor enhanced phosphorylation of p85 is responsible for the higher basal glucose uptake.

5.3.4 Cardioprotective properties of Diavite^{TM} in the ischaemic heart

Obesity has been linked to an increased risk of cardiovascular diseases, due to the multitude of risk factors associated with obesity, such as hypertension, insulin resistance, diabetes and dyslipidaemia [Reaven 1988, Seidell *et al.*, 1996; Lopaschuk *et al.*, 2007]. However, not many studies have investigated the susceptibility of an insulin resistant myocardium in the absence of diabetes to ischaemia/reperfusion injury. The focus is more on the susceptibility of a diabetic myocardium to ischaemia/reperfusion injury. However, it is likely that hearts from obese, insulin resistant individuals are more prone to ischaemia/reperfusion injury compared to non-obese individuals. A study done by Yue and colleagues (2005) showed that Zucker rats (*fa/fa*), treated with rosiglitazone, had an improved cardiac insulin sensitivity and that this increased insulin sensitivity was accompanied by an increased resistance to ischaemia/reperfusion injury.

In our model, hearts of the DIO animals at 8 and 12 weeks, were less susceptible to an ischaemic insult, which was contrary to expectations, as it does seem as if metabolic derangements start to occur as early as 8 weeks [Huisamen *et al.*, 2007 (unpublished data)]. In this study, done in our laboratories by Huisamen and colleagues (2007), the early effects of DIO on myocardial function were assessed. They found that 8 weeks animals displayed signs of insulin resistance with significantly elevated blood glucose and serum insulin levels, as well as an elevation in body weight. This is similar to what we observed in the present study. In addition, their obese animals showed significantly elevated plasma triglycerides, phospholipids and lipid peroxidation levels. This observation was also accompanied by a significant decrease in aortic output recovery after global ischaemia. It would seem logical that the infarct size would be greater in the DIO animals compared to their controls, as obesity does not occur in isolation from other metabolic

disorders that cumulatively lead to cardiac dysfunction. This is however not the case in our study, the study by Huisamen and colleagues (2007), Thim and colleagues (2006) and Kristiansen and colleagues (2004) all experienced the same unexpected occurrence. There is currently no known evidence as to what causes this.

We also found that Diavite™ treatment significantly decreased the infarct size in our 16 week DIO as well as control animals (refer to Figure 4.1.3). It might be that Diavite™ has an effect on proteins such as PKB/Akt and PI3K, which are known to be activated through the pro-survival pathway [Duronio, 2008; Baines *et al.*, 1999]. Acute activation of the serine-threonine kinase PKB/Akt is cardioprotective and increases glucose uptake, at least in part, through enhanced expression of GLUT4 [Matsui *et al.*, 2006]. The pathway that involves PI3K and PKB/Akt appears to both negatively regulate factors that promote the expression of death genes and positively regulate factors that induce survival [Duronio, 2008; Parcellier *et al.*, 2008]. Thus, Diavite™ might induce expression of anti-apoptotic factors, such as Bcl-2 while suppressing expression of pro-apoptotic factors, such as caspase-9. However, this was not investigated in our study and warrants further investigation. Another possibility for the observed cardioprotection in our study might be the increased serum insulin levels observed in both our models. Diavite™ treatment lead to increased β-cell formation, which was the reason for the increased serum insulin levels we observed and this increased insulin might be the reason for the increased protection observed, as insulin itself is a mitogen and is known to promote cell survival [Ryu *et al.*, 1999].

5.4 Adverse effects of Diavite™ consumption

In the absence of published data on this plant product, there is also not much known about the adverse effects. A standard toxicology study was conducted at the primate unit of the MRC (Cape Town) under the supervision of Dr. Jurgen Seier, to determine the side effects, if any, of over-consumption of this product. It was found that after treating Vervet monkeys with a 1x, 5x and 25x

therapeutic dose, no clinically relevant changes were observed. In addition, the monkeys did not show signs of hypoglycaemia at any stage over the 3 month experimental period. Data were compiled in a 100-page document, which is available on request. This data is not shown, as the report received on the results obtained was too lengthy.

CHAPTER 6
Conclusion and future research

The present study has shown that Diavite^{TM}, which is the trade name of the product produced from the pods of the *P. Glandulosa* tree, modestly lowers fasting blood glucose levels, stimulates insulin secretion and leads to the formation of small β-cells in rat models. In addition, this study has shown that orally consumed Diavite^{TM} elicits cardioprotection, by protecting the heart after an ischaemic incident, as seen through the presence of decreased infarct sizes. It was also evident that Diavite^{TM} treatment could improve glucose uptake by isolated cardiomyocytes, which is an indication of improved insulin sensitivity. Furthermore, it has been established that Diavite^{TM} treatment has no obvious detrimental effects in either of our rat models and no short-term toxic effects or hypoglycaemia over a 3 month period in Vervet monkeys (data not shown).

Future research

To our knowledge, no studies have been conducted on the mechanisms involved in the effects of this plant product and thus the active component of Diavite^{TM} has not yet been identified. Due to the multitude of promising results, such as the modest lowering of glucose levels, increased insulin stimulation, decreased infarct size (after an ischaemic incident), increased insulin sensitivity and β-cell regenerative capacity, observed with Diavite^{TM} consumption, it might be feasible to identify and extract the active component(s) and package it in the form of capsules instead of the raw plant material. As mentioned, Diavite^{TM} consumption elicits multiple effects in our animal models, thus isolating the active components can help determine whether the effects observed are as a result of one active substance or as a combination of many.

We suspect that the decreased infarct size observed in the DIO insulin resistant model could be due to the cardioprotective mechanisms, which are initiated after insulin binding. Thus, we assume the altered expression/activation of signaling molecules known to be involved in the insulin signaling pathway are responsible for the cardioprotection we observed in our rodent model. Therefore, by perfusing the hearts, as explained in section 2.3.1 of Materials and Methods, and determining the protein expression the signaling molecules involved in the mechanism of cardiac protection can be determined. We can also utilize an insulin-receptor-knockout model and determine infarct size, similar to our DIO insulin resistant model. With this model we will be able to evaluate whether the observed decreased infarct size are indeed via mechanisms pertaining to the insulin signaling pathway. To investigate this hypothesis we will utilize the cardiomyocyte-selective insulin receptor knockout (CIRKO) mice, which are obtainable from the laboratories of Prof Dale Abel (University of Utah, Salt Lake City, USA) for our analysis.

Another avenue to be explored is the regenerative capacity of Diavite^{TM}. From our analysis it is not clear what the origin of these small β-cells are and why Diavite^{TM} treatment increases the amount of β-cell formation. Thus, for future research, it might be feasible to determine the expression of certain transcription factors, such as Ngn3, MafA and Pdx1 in pancreata from Diavite^{TM} treated animals. We can also investigate the origin of these small β-cells, which might provide a better understanding as to what the role of Diavite^{TM} is in this situation.

Thus, given the current evidence, it seems as if Diavite^{TM}, after short-term use, is beneficial on multiple levels as a dietary supplement in both type 1 and type 2 diabetes, however further research is necessary.

CHAPTER 7

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